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Vasoactive Intestinal Peptide and Pituitary Adenylate Cyclase-Activating Polypeptide Inhibit Chemokine Production in Activated Microglia

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ABSTRACT Microglia react to even minor disturbances in CNS homeostasis and function as critical regulators of CNS inflammation. Activated microglia secrete inflammatory mediators such as cytokines and chemokines, which contribute to the pathophysiological changes associated with several neuroimmunologic disorders. Microglia-derived inflammatory chemokines recruit various populations of immune cells, which initiate and maintain the inflammatory response against foreign antigens. Entry and retention of activated immune cells in the CNS is a common denominator in a variety of traumatic, ischemic, and degenerative diseases. Vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating polypeptide (PACAP) are two structurally related neuropeptides that function as potent anti-inflammatory factors in the periphery. Here we investigated the effects of VIP and PACAP on chemokine production by activated microglia. VIP and PACAP inhibit the expression of the microglia-derived CXC chemokines MIP-2 and KC, and of the CC chemokines MIP-1α, -1β, MCP-1, and RANTES. The inhibition of chemokine gene expression correlates with an inhibitory effect of VIP/PACAP on NFkB binding. The VIP/PACAP inhibition of both chemokine production and of NFkB binding is mediated through the specific receptor VPAC1 and involves a cAMP-dependent intracellular pathway. Of biological significance is the fact that the inhibition of chemokine production by VIP/PACAP leads to a significant reduction in the chemotactic activity generated by activated microglia for peripheral leukocytes, i.e., neutrophils, macrophages, and lymphocytes. Because reduction in the number and activation of infiltrating leukocytes represents an important factor in the control of inflammation in the CNS, VIP and/or PACAP released by neurons during an inflammatory response could serve as neuronal survival factors by limiting the inflammatory process. *GLIA 39:148–161, 2002.*

INTRODUCTION

Entry and retention of activated immune cells in the CNS is a common denominator in a variety of traumatic, ischemic, and degenerative diseases. These cellular events are determined and controlled by a network of chemokines, adhesion molecules, and cytokines. In the CNS, resident microglia represent one of the most potent sources for proinflammatory products. Microglia are readily activated in most neuropathological conditions, and the activation process leads to changes in morphology, cell surface receptors, expression of cytokines and chemokines (Streit et al., 2000). Chemokines, small molecular weight (MW) polypeptides, are

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among the earliest secreted products, function as chemoattractants for cells expressing the appropriate chemokine receptors, and are responsible for the directional migration of blood-derived leukocytes to the CNS. Proinflammatory chemokines have been associated with various neuropathological conditions such as Alzheimer's disease, stroke, AIDS dementia, and particularly multiple sclerosis (MS). In patients with MS, increases in the expression of monocyte chemoattractant protein (MCP-1; CCL2), macrophage inflammatory protein (MIP-1α; CCL3), IFNγ-inducible protein 10 (IP-10; CXCL10), and RANTES (CCL5) are associated with demyelinating lesions and clinical neurological dysfunctions (Zhang et al., 2000). In addition, in experimental allergic encephalomyelitis (EAE), increases in MCP-1 and MIP-1α mRNA precede the onset of the disease, and treatment with either anti–MIP-1α or anti–MCP-1 Abs leads to a reduction in clinical symptoms (Godiska et al., 1995; Karpus and Kennedy, 1997; Karpus and Ransohoff, 1988).

The CNS consists of several cell types, which obviously interact both directly and through secreted products. In terms of consequences for the initiation and outcome of an inflammatory process, the interactions between these different cell types are not understood. Some of the neuronal-released neuropeptides were reported to affect glial cell activation. For example, somatostatin inhibits microglia proliferation (Feindt et al., 1998), vasoactive intestinal peptide (VIP) inhibits IFNα-induced MHC class II expression and synergizes with IL-1β to stimulate IL-6 production in astrocytes (Frohman et al., 1988; Gottschall et al., 1994), substance P increases, and α-melanocyte stimulating hormone (α-MSH), adrenocorticotropin (ACTH), VIP, and pituitary adenylate cyclase-activating polypeptide (PACAP) inhibit TNFα secretion from LPS-stimulated microglia (Luber-Narod et al., 1994; Galimberti et al., 1999; Kim et al., 2000). However, with the exception of morphine, which was shown to inhibit RANTES (CCL5) production from LPS- and IL-1β–stimulated microglia (Hu et al., 2000), there is no information regarding the effects of neuropeptides on chemokine production by activated microglia. Based on our previous report that the two structurally related neuropeptides, VIP and PACAP, inhibit proinflammatory chemokine production in macrophages (Delgado and Ganea, 2001a), we investigated their potential role in regulating chemokine production by activated microglia.

MATERIALS AND METHODS

Reagents

Synthetic VIP and PACAP38 were purchased from Calbiochem-Novabiochem (San Diego, CA). The PAC1/VPAC2 antagonist $PACAP_{6-38}$ was obtained from Peninsula Laboratories (Belmont, CA). The VPAC1 antagonist [Ac-His^1, D-Phe^2, K^{15}, R^{16}, L^{27}] VIP (3–7)-GRF (8–27) and the VPAC1 agonist [K^{15}, R^{16}, L^{27}] VIP (1–7)-GRF (8–27) were kindly donated by Dr. Patrick Robberecht (Universite Libre de Bruxelles, Belgium). The VPAC2 agonist Ro 25–1553 Ac-[Glu^8, Lys^{12}, Nle^{17}, Ala^{19}, Asp^{25}, Leu^{26}, $Lys^{27,28}$, $Gly^{29,30}$, Thr^{31}]-VIP cyclo (21–25) was a generous gift from Drs. Ann Welton and David R. Bolin (Hoffmann-La Roche, Nutley, NJ). The synthetic PAC1 agonist maxadilan was a generous gift from Dr. Ethan A. Lerner (Massachusetts General Hospital, Charlestown, MA). Murine recombinant TNFα, IFNγ, and IL-1β, and capture and biotinylated antibodies against JE/MCP-1 and MIP-1β murine were purchased from Pharmingen (San Diego, CA). Capture and biotinylated antibodies against murine KC, RANTES, MIP-2, and MIP-1α were purchased from PreproTech (Rocky Hill, NJ). LPS (from *E. coli* 055:B5), protease inhibitors, db-cAMP, and forskolin were purchased from Sigma Chemicals (St. Louis, MO), and N-[2-(p-bromocinnamyl-amino)ethyl]-5-iso-quinolinesulfonamide (H89) from ICN Pharmaceuticals (Costa Mesa, CA). Recombinant IkBα (1-317) and antibodies against p65, p50, IkBα, phosphorylated-IkBα, IkB-kinase (IKKα) and CREB were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Cultures

Microglial cell cultures were prepared as previously described (Chao et al., 1993). Briefly, cerebral cortical cells from 1-day-old Balb/c mice were dissociated by trypsinization (0.25% trypsin, 30 min) and were plated in 75 cm^2 Falcon culture flasks in Dulbecco's modified Eagle's medium (DMEM) high-glucose formula (Life Technologies) supplemented with 10% heat-inactivated fetal calf serum (FCS; Gibco-BRL), containing 10 mM HEPES buffer, 1 mM pyruvate, 0.1 M nonessential aminoacids, 2 mM glutamine, 50 mM 2-mercaptoethanol, 100 U/ml penicillin, and 10 μg/ml streptomycin (complete medium). The medium was replenished 1 and 4 days after plating, and on day 8 the plates were shaken for 20 min at a speed of 200 rpm in an orbital shaker to remove oligodendrocytes. On day 12, the plates were shaken again for 2 h at a speed of 180–200 rpm. The harvested cells were filtered through a 20 μm nylon mesh, plated in 60 mm Petri dishes, and incubated for 15 min at 37°C. After extensive washing with culture medium, adherent cells (microglia) were collected with a rubber policeman and centrifuged (1000 rpm, 10 min). The purified microglial cells were $> 98\%$ MAC-1^+ and $< 2\%$ $GFAP^+$ specific. Two mouse microglial cell lines (EOC13 and BV2) were used in some experiments. The EOC13 cell line was maintained as previously described (Walker et al., 1995) in DMEM complete medium containing 2 mM glutamine, 10% heat-inactivated FCS, and 20% LADMAC-conditioned medium. The BV2 murine microglial cell line generated by Blasi et al. (1990) was maintained in DMEM high glucose supplemented with 5% heat-inactivated FCS, 4 mM L-glutamine, 0.2 mM penicillin, 0.05 mM streptomycin, and 20 mM HEPES.

Microglia (murine primary microglia, EOC13, and BV2 cells) were incubated with complete medium and stimulated with 100 ng/ml LPS in the presence or absence of VIP or PACAP38 (from 10^{-12} to 10^{-6} M). Cell-free supernatants were harvested at designated time points and kept frozen (−20°C) until chemokine determination by ELISA.

Mouse peritoneal exudate cells (PECs) were obtained by peritoneal lavage with ice-cold RPMI 1640 medium from male Balb/c mice (aged 6–10 weeks). PECs, containing polymorphonuclear cells (PMN), lymphocytes, and macrophages, were washed twice and resuspended in ice-cold RPMI 1640 medium supplemented with 2% heat-inactivated FCS, containing 10 mM HEPES buffer, 1 mM pyruvate, 0.1 M nonessential aminoacids, 2 mM glutamine, 50 mM 2-mercaptoethanol, 100 U/ml penicillin, and 10 μg/ml streptomycin.

RT-PCR for Detection of VPAC1, VPAC2, and PAC1 mRNA Expression

Microglia cells were cultured at a concentration of 2×10^6 cells/ml in 100 mm tissue culture dishes and stimulated with LPS (0.5 μg/ml) for up to 12 h. Cells were collected at different time points (0 and 12 h) and total RNA was isolated using the Ultraspec RNA reagent (Biotecx, Houston, TX) as recommended by the manufacturer; 2 μg of total RNA was reverse-transcribed in the presence of 200 units of MMLV-RT, 40 units of RNasin, 1 μg of random primers, 0.5 mM dNTPs, 3 μg of BSA, and MMLV reaction buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$) in a total volume of 30 μl at 37°C for 1 h.

The cDNA was then amplified with specific primers. Amplification with GADPH primers (Stratagene) was used as a control. The primers for VPAC1, VPAC2, and PAC1 receptors have been described before and have the following sequence: VPAC1 sense 5′-CCTTCTTCTCTGAGCGGAAGTACTT-3′ and antisense 5′-CCTGCACCTCGCCATTGAGGAAGCAG-3′; VPAC2 sense 5′-GTCAAGGACAGCGTGCTCTACTCC-3′ and antisense 5′-CCCTGGAAGGAACCAACACATAAC-3′; PAC1 sense 5′-CAAGAAGGAGCAAGCCATGTGC-3′ and antisense 5′-CATCGAAGTAATGGGGGAAGGG-3′; GAPDH sense 5′-TCCTGCACCACCAACTGCTTAGCC-3′ and antisense 5′-GTTCAGCTCTGGGATGACCTTGCC-3′. The expected sizes for the amplified fragments are 453 bp for VPAC1, 572 bp for VPAC2, 317 bp for PAC1, and 225 bp for GAPDH; 5 μl of reverse-transcribed cDNA was subjected to PCR in the presence of 0.5 units of pyrostase, 1 μM sense and antisense primers, 0.2 mM dNTPs, and polymerase buffer (50 mM Tris-HCl, pH 9.0, 1.5 mM $MgCl_2$, 20 mM $(NH_4)_2SO_4$, 50 μg/ml BSA). The PCR conditions were denaturation at 94°C for 1 min, annealing at 60°C for 1 min, primer extension at 72°C for 2 min for 38 cycles. The PCR products were size-separated in 2% agarose gel and visualized by UV light.

Electrophoretic Mobility Shift Assay (EMSA)

BV2 cells were cultured at a density of 10^7 cells in six-well plates, stimulated as described above, washed twice with ice-cold PBS/0.1% BSA, and harvested. Nuclear extracts were prepared by the mini-extraction procedure of Schreiber et al. (1989). Double-stranded oligonucleotides (50 ng) corresponding to the NF-kB sites from murine MCP-1 (5′-ACTGCCCTCAGAATGGGAATTTCCACGCTCTTATC-3′) (Freter et al., 1992), RANTES (5′-TTTTGGAAACTCCCCTTAGGGGATGCCCCTCA-3′) (Nelson, 1993), MIP-2 (5′-CCCTGAGCTCAGGGAATTTCCCTGGTCCCCG-3′) (Widmer et al., 1993), and KC (5′-TACTCCGGGAATTTCCCTGGCC-3′) (Ohmori et al., 1995) were end-labeled with γ^{32}P-ATP. The binding reaction mixtures (15 μl) were set up as follows: 0.5–1 ng DNA probe (20,000–50,000 cpm), nuclear extract (5 μg protein), 2 μg poly(dI-dC).poly(dI-dC), and binding buffer (50 mM NaCl, 0.2 mM EDTA, 0.5 mM DTT, 5% glycerol, and 10 mM Tris-HCl, pH 7.5). The mixtures were incubated on ice for 15 min before adding the probe, followed by 20 min at room temperature. Samples were loaded on 4% nondenaturing polyacrylamide gels and electrophoresed in TGE buffer (50 mM Tris-HCl, pH 7.5, 0.38 M glycine, and 2 mM EDTA) at 100 V, followed by transfer to Whatman paper, and autoradiography. In competition and antibody supershift experiments, the nuclear extracts were incubated for 15 min at room temperature with the specific antibody (1 μg), or with competing cold oligonucleotide (50-fold excess) before the addition of the labeled probe.

Chemokine ELISA

The content of chemokines in the culture supernatants was determined by specific sandwich ELISAs as previously described (Delgado and Ganea, 2001a). The cytokine amount in each supernatant was extrapolated from standard curves. The lower limit of detection for JE/MCP-1, MIP-1α, MIP-1β, MIP-2, RANTES, and KC was 16, 5, 7, 8, 10, and 15 pg/ml, respectively.

VIP Elisa

VIP concentrations were determined by using a specific competitive ELISA as previously described (Martinez et al., 1999).

Ribonuclease Protection Assay (RPA) for Detection of Chemokine mRNA Expression

Murine primary microglia or microglia cell lines were cultured at a concentration of 2×10^6 cells/ml in 100 mm tissue culture dishes and stimulated with LPS (100 ng/ml) in the presence or absence of VIP (10^{-8} M) or PACAP (10^{-8} M) for up to 12 h. Total RNA was

isolated using the Ultraspec RNA reagent (Biotecx, Houston, TX) as recommended by the manufacturer. RNase protection assays (RPA) were performed with 2.5–5 μg RNA using the Riboquant MultiProbe RNase Protection Assay System (PharMingen) according to the manufacturer's instructions. Each commercial kit contained a set of chemokine templates as well as a template for the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The samples were electrophoresed on 5% denaturing polyacrylamide gels. Signal quantitation was performed in a PhosphorImager SI (Molecular Dynamics, Sunnyvale, CA).

Western Blotting

Whole cell lysates, cytoplasmic fractions, or nuclear extracts containing 20 to 30 μg of protein were subjected to reducing SDS-PAGE (12.5%). After electrophoresis, the gel was electroblotted and the membranes were with primary antibodies (rabbit antimouse IgG against IkBα, IKKα, NF-kB p50, or NF-kB p65, or mouse IgG against phosphorylated-IkBα, at dilutions ranging from 1:500 to 1:2,000), followed by secondary antibodies, peroxidase-conjugated goat antirabbit IgG or rat antimouse IgG (1:5,000 dilution). The membranes were developed by using the enhanced chemiluminiscence detection system (ECL, Amersham).

In Vitro Kinase Assay

The in vitro IKKα kinase assay was performed as previously described (Delgado and Ganea, 2001b). Whole cell lysates were prepared from 2×10^6 cells and cleared by centrifugation at 13,000 g for 3 min. The endogenous IKKα was immunoprecipitated by incubating the lysates (150–250 μg protein) with 0.5 μg of anti-IKKα antibody for 2 h at 4°C. The immune complexes were collected by incubation with protein A/G-Sepharose beads for 45 min at 4°C. Following extensive washing, the pelleted beads were resuspended in 30 μl kinase buffer containing 15 μM ATP, 10 μCi [γ^{32}P]ATP (3,000 Ci/mmol), and 5 μg of recombinant IkBα. The kinase reaction was performed at 30°C for 30 min and stopped by the addition of 15 μl of 2 × SDS sample buffer. Following boiling for 5 min, the samples were subjected to SDS-PAGE (9%). Proteins were transferred on nitrocellulose membranes, followed by autoradiography and quantitation of radioactive IkBα by phosphoimaging. Presence of IKKα protein was verified by immunoblotting.

Chemotactic Activity of Microglial Conditioned Medium

Cell migration of peritoneal cell populations was evaluated using a 48-well chemotaxis microchamber, as previously described (Meda et al., 1996). Briefly, dilutions of supernatants from microglia cultures were added to the lower wells of the chemotaxis chamber (Neuroprobe, Pleasanton, CA). Complete medium was used as basal control. A polycarbonate filter (5 μm pore size, Neuroprobe) was layered onto the wells and covered with a silicon gasket and the top plate. Freshly isolated peritoneal cells (1.5×10^6 cells/ml) were seeded in the upper chamber. Incubation was conducted at 37°C for 90 min. The filters were removed, stained with May-Grunwald and Giemsa, and the number of macrophages, lymphocytes, and PMN that had migrated was evaluated by counting eight microscopic fields (oil immersion) and distinguished based on morphology.

Statistics

Comparison between groups were made using the student's *t*-test followed by Scheffe's F-test, with $P < 0.001$ as the minimum significant level.

RESULTS

VIP and PACAP Inhibit LPS-Induced Production of CXC and CC Chemokines in Microglia

Primary microglia were activated with LPS in the absence or presence of various doses of VIP or PACAP, and the amounts of chemokines released in culture supernatants were assayed by ELISA. Unstimulated microglia produce very low amounts of chemokines (Fig. 1A). LPS stimulation results in a time-dependent increase in the production of the CXC chemokines MIP-2 and KC (murine neutrophil attractants), and of the CC chemokines MIP-1α (CCL3), MIP-1β (CCL4), MCP-1 (CCL2), and RANTES (CCL5), with peak levels at 8 to 24 h (Fig. 1A). Chemokine levels declined only slowly after 48 h. VIP and PACAP inhibit in a dose- and time-dependent manner the production of the CXC and CC chemokines tested (Fig. 1). Chemokine production was significantly inhibited as early as 2 h, with maximum inhibitory effects at 24 to 48 h (Fig. 1A). The reduction of chemokine production was maintained throughout 72 h (not shown), indicating that VIP and PACAP do not delay, but rather reduce chemokine release. The dose-response curves were similar for VIP and PACAP, with maximal effects at 10^{-8} M (Fig. 1B).

The inhibitory effects were not the result of a decreased number of microglial cells, as neither VIP nor PACAP affected cell numbers or the viability of stimulated microglia after 36 h of culture (viabilities determined by Trypan blue exclusion were in the range of 87%–92% with or without neuropeptides).

In addition, VIP and PACAP also inhibit chemokine production following activation with proinflammatory cytokines (i.e., TNFα, IL-1β, and IFNγ; Fig. 2). Since the highest degree of inhibition was observed for mi-

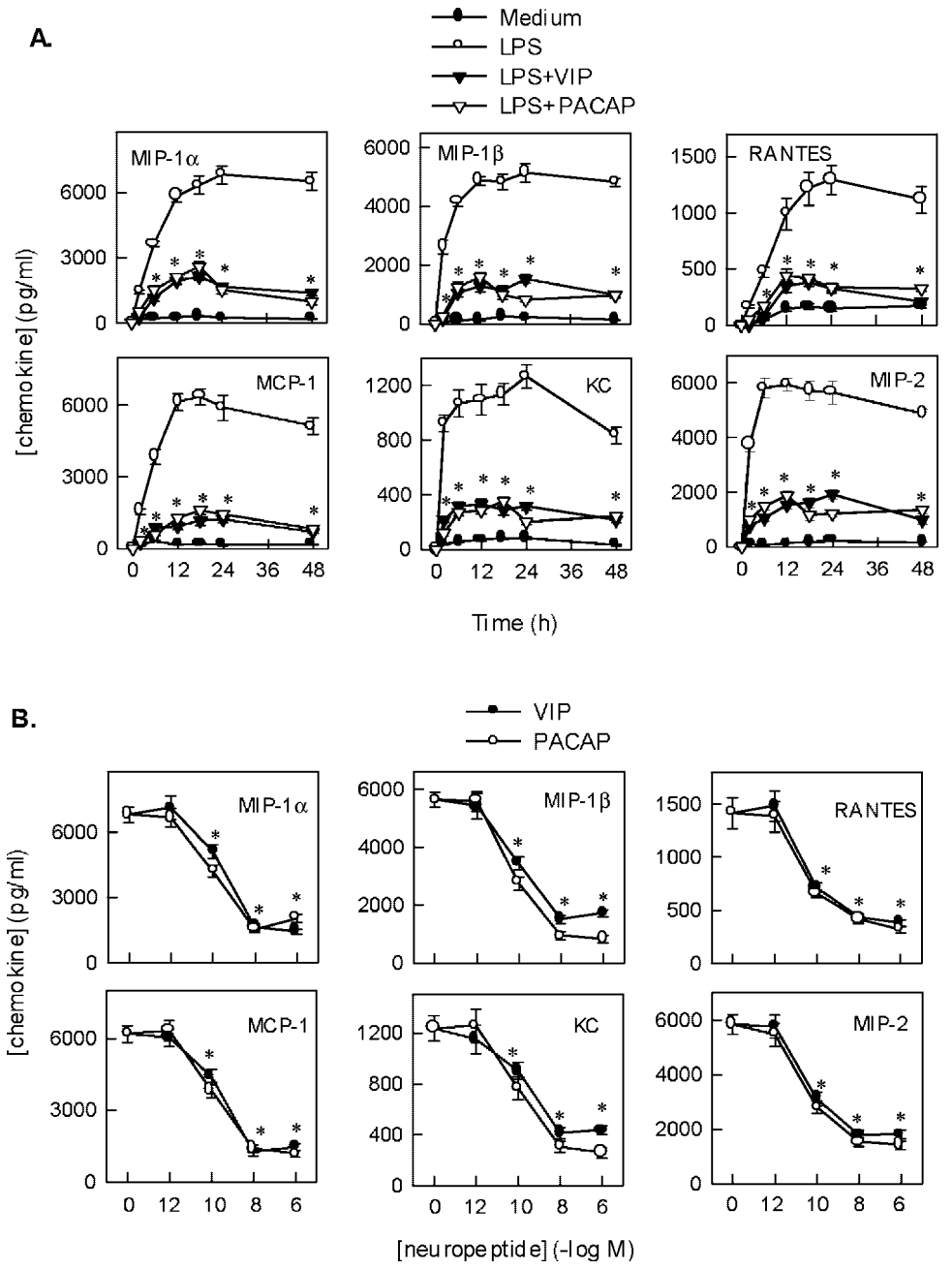

Fig. 1. VIP and PACAP inhibit LPS-induced chemokine production. **A:** Time-dependent inhibition. Primary microglia (2×10^5 cells/ml) were cultured in 48-well plates (1 ml/well) and stimulated with LPS (100 ng/ml), in the absence or presence of 10^{-8} M VIP or PACAP. Supernatants harvested at different time points were assayed for chemokine content by ELISA. **B:** Dose-dependent inhibition. Primary microglia were stimulated with LPS and treated with various concentrations of either VIP or PACAP for 24 h. The chemokine contents in the culture supernatants were determined by ELISA. Each result is the mean ± SD of four separate experiments performed in duplicate.

croglia stimulated with 100 ng/ml LPS at a neuropeptide concentration of 10^{-8} M after 24 h of culture, we used these conditions for the rest of the experiments.

Inhibition of Chemokine Production by VIP and PACAP Is Mediated Through VPAC1

Next we investigated whether the inhibitory effect of VIP/PACAP is mediated through specific receptors. VIP and PACAP act through a family of receptors consisting of VPAC1, VPAC2, and PAC1 (Harmar et al., 1998). RT-PCR analysis indicates that murine primary microglia express both PAC1 and VPAC1 mRNA (Fig. 3A). In contrast, VPAC2 mRNA was not expressed even following LPS activation (Fig. 3A). A similar pattern of VIP/PACAP receptor expression was observed in rat primary microglia (Kim et al., 2000) and in the two murine microglial cell lines EOC13 and BV2 (Fig.

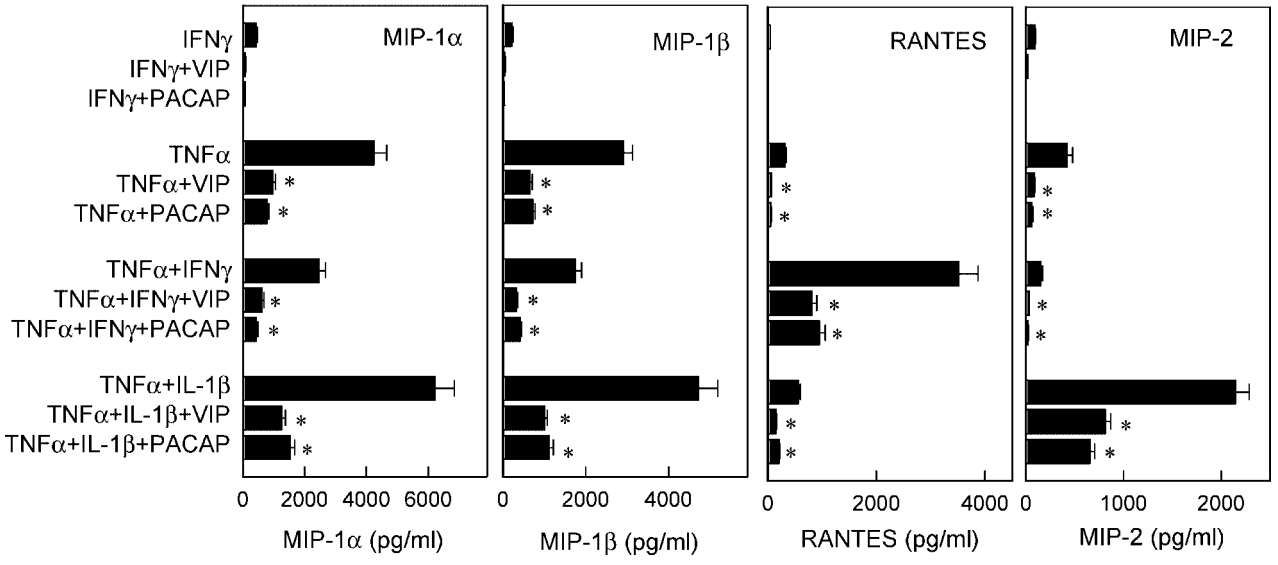

Fig. 2. VIP and PACAP inhibit cytokine-induced chemokine production. Murine primary microglia were stimulated with TNFα (20 ng/ml), IFNγ (250 U/ml), TNFα plus IFNγ, or TNFα plus IL-1β (250 U/ml), in the absence or presence of 10^{-8}M VIP or PACAP, and supernatants harvested at different time points were assayed for chemokine production by ELISA. Each result is the mean ± SD of three separate experiments performed in duplicate.

3B). In order to determine which of the VIP/PACAP receptors is involved in the inhibition of chemokine production, we used specific receptor agonists and antagonists. The effects of the VPAC1 agonist [K^{15}, R^{16}, L^{27}] VIP (1–7)-GRF (8–27) (Gourlet et al., 1997a), the VPAC2 agonist (Ro 25–1553) (Xia et al., 1997), and of maxadilan, a PAC1 agonist (Moro and Lerner, 1996), were tested. The VPAC1 agonist, but not the VPAC2 or the PAC1 agonist, inhibits the release of chemokines, with a potency similar to that of VIP/PACAP (Fig. 3C). In addition, we investigated the ability of $PACAP_{6-38}$, a PAC1 antagonist that also binds with less affinity to VPAC2 (Gourlet et al., 1995), and of the specific VPAC1 antagonist [Ac-His^1, D-Phe^2, K^{15}, R^{16}, L^{27}] VIP (3–7)-GRF (8–27) (Gourlet et al., 1997b), to reverse the effects of VIP and PACAP. Increasing concentrations of the antagonists (10^{-8} to 10^{-5} M) were added simultaneously with 10^{-8} M VIP or PACAP. The VPAC1 antagonist reversed the effects of VIP/PACAP in a dose-dependent manner (Fig. 3D). In contrast, $PACAP_{6-38}$ did not reverse the inhibitory effect (Fig. 3D). Together, these results indicate that both neuropeptides exert their action primarily through VPAC1.

Intracellular Signal Pathways Involved in Inhibitory Effect of VIP and PACAP on Chemokine Production

To determine whether intracellular cAMP acts as a secondary messenger, we determined the effects of H89 (a PKA inhibitor), calphostin C (a PKC inhibitor), forskolin (a cAMP-inducing agent), and db-cAMP (a cAMP analog). Forskolin and db-cAMP inhibit chemokine release similar to VIP and PACAP (Fig. 4A). In addition, H89, but not calphostin C, completely reverses the inhibitory effect of VIP/PACAP (Fig. 4B). These results suggest that the inhibitory effect of VIP/PACAP is mediated through increases in intracellular cAMP.

VIP and PACAP Inhibit Chemokine Production at mRNA Level

Primary microglia were treated with LPS in the presence or absence of 10^{-8} M VIP or PACAP for 2, 6, 12, 18, and 24 h; total RNA was prepared and subjected to RPA analysis. Although no or very little chemokine mRNA is detectable in unstimulated cells (Fig. 5), progressively increased levels of MIP-2, MIP-1α, MIP-1β, MCP-1, and RANTES mRNA are present in LPS-stimulated cells (maximal at 6–12 h; Fig. 5). At all time points, VIP and PACAP significantly inhibited the levels of chemokine mRNA (Fig. 5). These results indicate that the two neuropeptides significantly reduce MIP-2, MIP-1α, MIP-1β, MCP-1, and RANTES steady-state mRNA levels. A similar pattern of chemokine mRNA inhibition was observed in EOC13 and BV2 cells (Fig. 6).

VIP and PACAP Prevent NFkB Binding to Chemokine Promoters and Inhibit Subsequent NFkB-Dependent Gene Activation

Although the promoters of most chemokines contain complex arrays of transactivating binding sites, NFkB appears to be essential for maximal chemokine transcription following LPS stimulation (Freter et al., 1992, 1995, 1996; Grover and Plumb, 1993; Nelson et al., 1993; Widmer et al., 1993; Ohmori et al., 1995). In

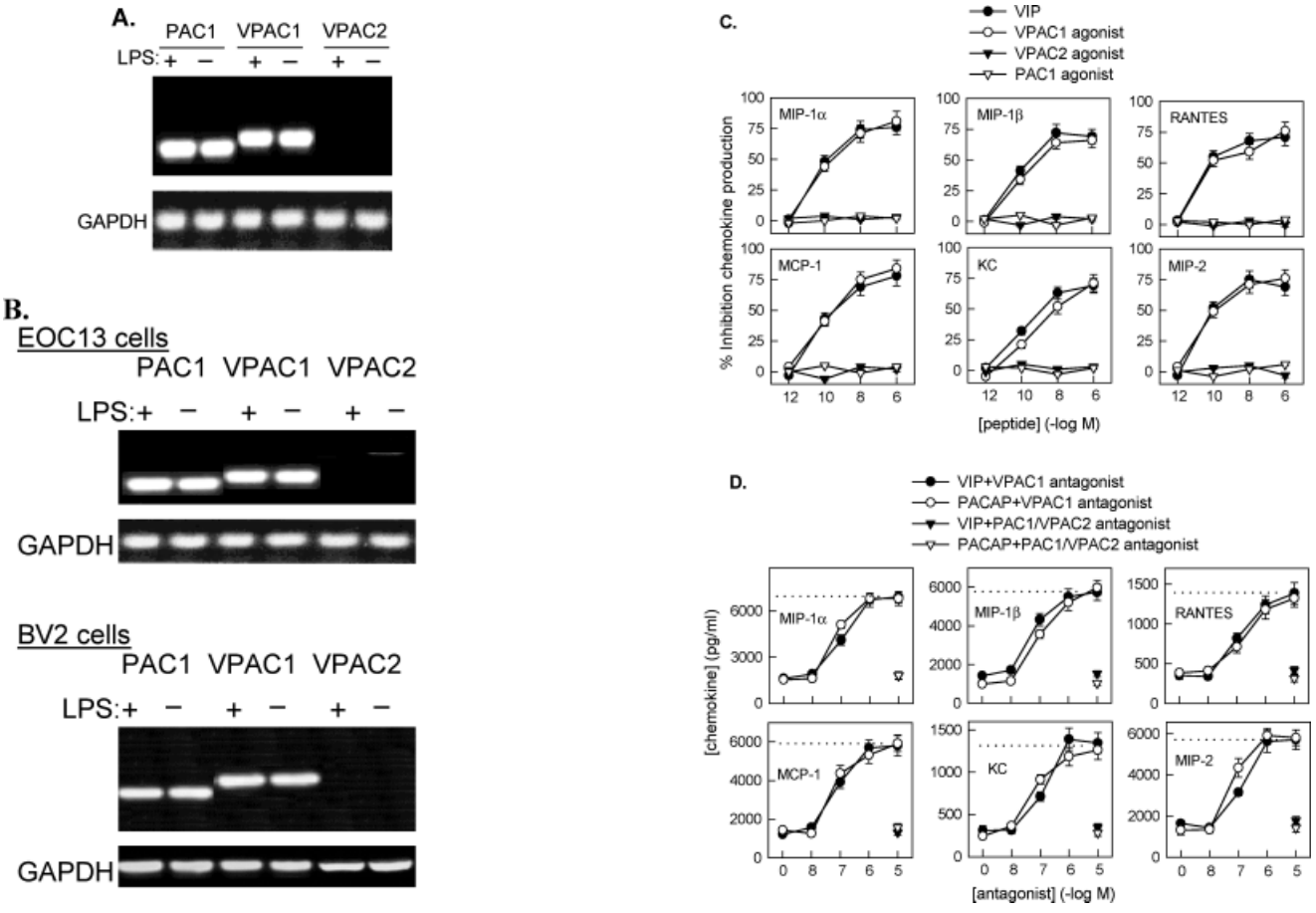

Fig. 3. The effect of VIP/PACAP is mediated through VPAC1. **A:** Expression of VPAC1, VPAC2, and PAC1 mRNA in murine microglia. Total RNA extracted from unstimulated and LPS-stimulated (12 h) primary microglia (10^7 cells) was subjected to RT-PCR with specific primers for VPAC1, VPAC2, and PAC1. One representative experiment of two is shown. **B:** Expression of VPAC1, VPAC2, and PAC1 mRNA in microglia cell lines. One representative experiment of two is shown. **C:** Comparative effects of VIP/PACAP receptor agonists on chemokine production. Primary microglia were stimulated with LPS in the presence or absence of different concentrations of VIP, maxadilan (a PAC1 agonist), Ro 25–1553 (a VPAC2 agonist), and [K^{15}, R^{16}, L^{27}] VIP (1–7)-GRF (8–27) (a VPAC1 agonist). Supernatants collected 24 h later were assayed for chemokine production by ELISA. Percentage inhibition was calculated by comparison with controls containing LPS alone. Each result is the mean ± SD of four experiments. Each sample was assayed in duplicate. **D:** Effect of PAC1 and VPAC antagonists. Primary microglia were stimulated with LPS and treated simultaneously with VIP or PACAP (10^{-8}M), and different concentrations of the VPAC1 antagonist [Ac-His^1, D-Phe^2, K^{15}, R^{16}, L^{27}] VIP (3–7)-GRF (8–27), or the PAC1/VPAC2 antagonist ($PACAP_{6-38}$). Supernatants collected 24 h later were assayed for chemokine production by ELISA. The VPAC1 antagonist (10^{-6}M) and $PACAP_{6-38}$ (10^{-6}M) did not affect chemokine production in LPS-treated microglia. The dotted line represents control values from cultures incubated with LPS alone. Each result is the mean ± SD of four experiments performed in duplicate.

macrophages/monocytes, VIP and PACAP inhibit production of several proinflammatory cytokines by downregulating NFkB binding and transactivation (Delgado M et al., 1998, 1999a; Delgado and Ganea, 1999, 2001b). To investigate whether VIP and PACAP affect NFkB binding in microglia, we used the murine microglial cell line BV2. Stimulation of BV2 cells with LPS led to an increase in NFkB binding to KC, MIP-2, MCP-1, and RANTES promoters compared to unstimulated cells; in all cases, treatment with VIP and PACAP significantly inhibited the binding (Fig. 7A, left). The NFkB binding specificity was evident by the complete displacement of the NFkB/DNA binding complexes in the presence of a 50-fold excess of unlabeled kB oligonucleotides (Fig. 7A, middle). Antibody supershift experiments indicate that the NFkB-binding complexes consist primarily of p50/p65 heterodimers (Fig. 7A, right).

The primary level of control for NF-kB is mediated through its interaction with the inhibitor IkB. VIP and PACAP could inhibit NF-kB activity by blocking LPS-induced IkB degradation and subsequent NF-kB nuclear translocation. The levels of cytoplasmic and nuclear p65 were determined by Western blotting. As expected, p65 was predominantly localized in the cytoplasm of unstimulated cells, and LPS induced a decrease in cytoplasmic and an increase in nuclear p65 (Fig. 7B). VIP and PACAP abolished the LPS-induced change in p65 levels (Fig. 7B). Differences in p65 protein levels were not due to differences in protein loading since similar levels of p50 were detected regardless of treatment. To elucidate whether the VIP/PACAP

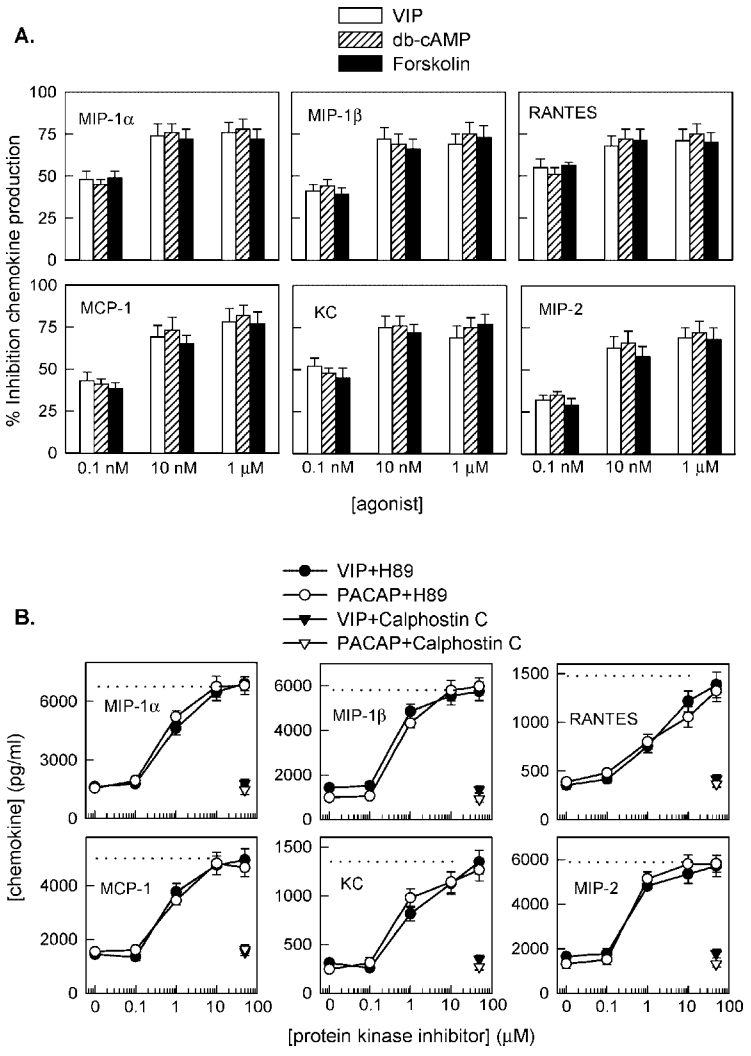

Fig. 4. Intracellular signal pathways involved in the VIP/PACAP inhibition of chemokine production. **A:** Effect of cAMP-inducing agents. Primary microglia were stimulated with LPS in the presence or absence of different concentrations of VIP, forskolin, or db-cAMP. Supernatants collected 24 h later were assayed for chemokine production. Percentage inhibition was calculated by comparison with control cultures incubated with LPS alone. Each result is the mean ± SD of five experiments performed in duplicate. **B:** Comparative effects of calphostin C (a PKC-inhibitor) and H89 (a PKA-inhibitor). Microglia were stimulated with LPS, incubated with or without VIP or PACAP (10^{-8} M), in the absence or presence of different concentrations of calphostin C or H89. Supernatants collected 24 h later were assayed for chemokine production. The dotted line represents control values from cultures incubated with LPS alone. Each result is the mean ± SD of four experiments performed in duplicate.

block in p65 nuclear translocation is caused by interference with the LPS-induced IkB degradation, we examined the cytoplasmic IkBα levels. In LPS-treated cells, we observed a time-dependent IkBα degradation, paralleled by an increase in IkBα phosphorylation (Fig. 7C). VIP and PACAP block the phosphorylation and subsequent degradation of IkBα (Fig. 7C).

Since IkB is phosphorylated by IKK kinases, we determined whether VIP and PACAP inhibit IKK activity in an in vitro kinase assay. Stimulation of BV2 cells with LPS resulted in a time-dependent increase in IKKα activity, which was inhibited by VIP and PACAP (Fig. 7D). No differences in IKKα expression were observed (Fig. 7D). These results demonstrate that VIP and PACAP inhibit the nuclear translocation and DNA binding of NF-kB by blocking the IKK-mediated IkB phosphorylation and subsequent degradation.

Receptors and Intracellular Pathways Involved in Effects of VIP and PACAP on NFkB

Since the inhibitory effect of VIP on chemokine production is mediated primarily through VPAC1 and cAMP, we determined the effect of the VPAC1 antagonist and of the PKA inhibitor H89 on the changes induced by VIP in kB-binding complexes and IKK activity. The VIP inhibition of NFkB binding, p65 nuclear translocation, and IkB phosphorylation was completely reversed by the VPAC1 antagonist (Fig. 8, lanes 3) and

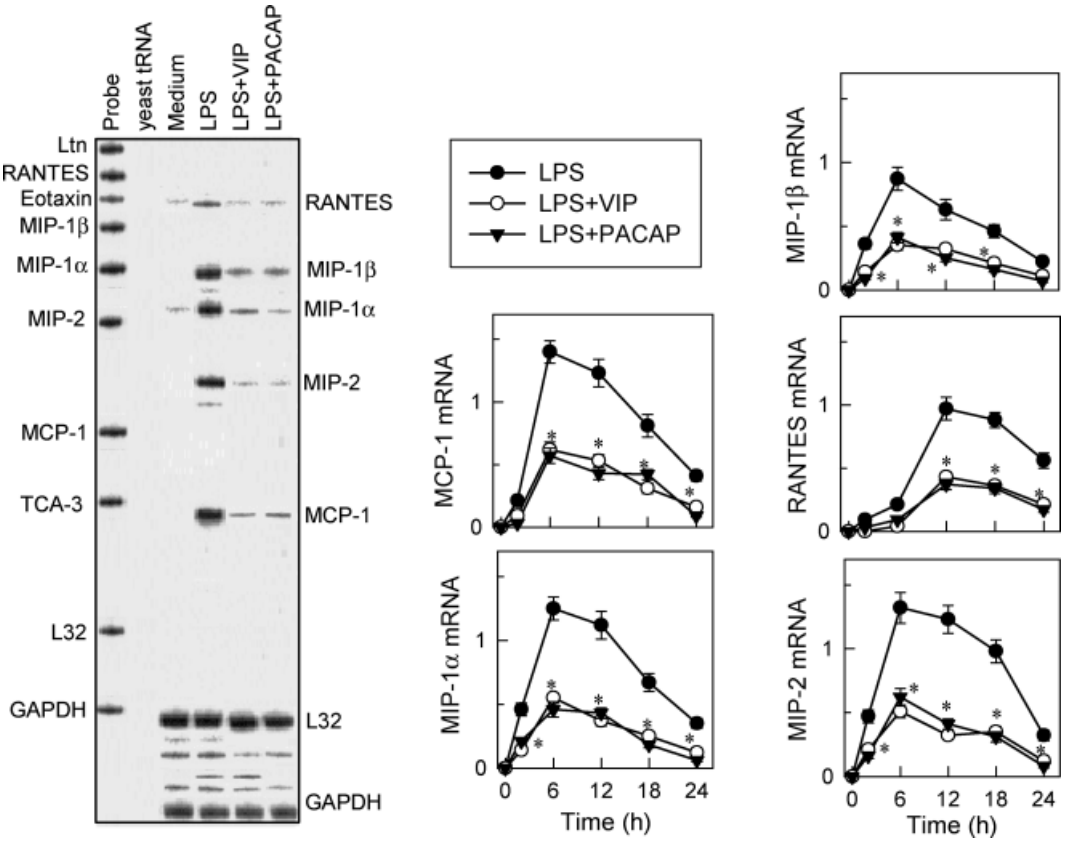

Fig. 5. VIP and PACAP inhibit LPS-induced chemokine mRNA expression. Primary microglia were stimulated with LPS in the presence or absence 10^{-8} M VIP or PACAP. At different time points (6 h for blot; left), the cells were lysed and subjected to RPA. Results (mean ± SD of four RPA assays performed on the same sample) are expressed in arbitrary densitometric units normalized for the expression of GAPDH in each sample.

by H89 (Fig. 8, lanes 4). These results suggest that the effects of VIP on NFkB are mediated through VPAC1 and are cAMP-dependent. This is supported by the fact that forskolin (a cAMP inducer) mimics the effect of VIP on NFkB binding, p65 nuclear translocation, and IKK activity (Fig. 8, lanes 5).

VIP and PACAP Inhibit Microglia-Induced Chemotactic Activity for Peripheral Leukocytes

Chemokines attract blood-derived leukocytes at sites of inflammation. Since VIP and PACAP inhibit endotoxin-induced chemokine production, we investigated next whether VIP and PACAP also inhibit chemotaxis. Conditioned medium from LPS-stimulated microglia was assayed for chemotactic activity. Supernatants from primary microglia cultured for 24 h in the presence of 100 ng/ml LPS exert chemotactic activity for murine peritoneal exudate cells (PECs), including neutrophils, macrophages, and lymphocytes (Fig. 9). Conditioned medium harvested from microglia cultured in the presence of VIP or PACAP induced a much lower chemotactic activity for all three types of PECs (Fig. 9). In control experiments, medium containing 10^{-8} M VIP or PACAP was incubated for 24 h at 37°C in the absence of microglia and tested for chemotactic activity. No effect was observed regarding the migration of PMNs, macrophages, or lymphocytes. In addition, no VIP was detected by Elisa in medium controls, suggesting that the neuropeptides are degraded after 24 h at 37°C. These results argue against a direct effect of VIP/PACAP on peripheral leukocytes and indicate a correlation between the VIP/PACAP inhibition of microglial chemokine production and the reduction in chemotactic activity for peripheral leukocytes.

DISCUSSION

Microglia, functional equivalents of mononuclear phagocytes, react to even minor disturbances in CNS homeostasis and function as critical regulators of CNS inflammation (Gonzalez-Scarano and Baltuch, 1999; Aloisi et al., 2000; Becher et al., 2000; Streit, 2000). Microglial activation was reported in a variety of neuropathological conditions, such as multiple sclerosis, Alzheimer's and Parkinson's disease, AIDS dementia, and spongiform encephalopaties (McGeer et al., 1988; Rogers et al., 1988; Matsumoto et al., 1992; Spencer

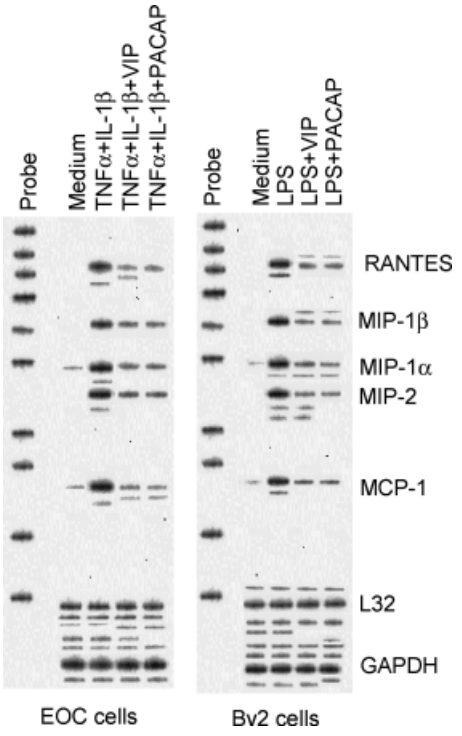

Fig. 6. VIP and PACAP inhibit chemokine mRNA expression in two microglia cell lines. BV2 and EOC13 cells (2×10^6 cells/ml) were stimulated with LPS (100 ng/ml) or with TNFα (20 ng/ml) plus IL-1β (250 U/ml), respectively, in the presence or absence of 10^{-8} M VIP or PACAP. Six hours later, the cells were lysed and subjected to RPA. Results are representative of three different experiments.

and Price, 1992; Giese and Kretzschmar, 2001; Glass and Wesselingh, 2001; Hoozemans et al., 2001; Rezaie and Lantos, 2001). Activated microglia secrete inflammatory mediators such as cytokines, chemokines, reactive oxygen, and nitrogen intermediates, which contribute to the pathophysiological changes associated with several neuroimmunologic disorders. Several studies have highlighted the link between chemokine production in the CNS and the type and intensity of the inflammatory response, due to the directional migration of blood leukocytes to the inflammatory site in the brain parenchyma (Eng et al., 1996; Bacon and Harrison, 2000; Luo et al., 2000; Tran et al., 2000; Zhang et al., 2000). Some chemokines such as SDF-1 (CXCL12) and fractalkine (CXCL1) are constitutively produced in brain and play a role in CNS development and homeostasis, whereas chemokines such as IL-8 (CXCL8), MIP-1α (CCL3), MIP-1β (CCL4), MCP-1 (CCL2), and RANTES (CCL5), generated by neuroinflammatory stimuli, contribute to the initiation of the inflammatory response through their chemotactic activity for neutrophils, monocytes/macrophages, and/or T-cells (Bagglioloni et al., 1997; Rollins, 1997; Luster, 1998; Maciejewski-Lenoir et al., 1999; Rossi and Zlotnik, 2000; Zlotnik and Yoshie, 2000).

In CNS, dynamic interplays occur among neurons and glial cells. Through the release of proinflammatory agents and the recruitment of blood-derived leukocyte, activated microglia affect neuronal survival and function. On the other hand, neurons may also control and regulate microglia activation and function, either directly or through mediators such as neuropeptides. The information regarding the effects of neuropeptides on microglial activation is sparse, and with one exception (Hu et al., 2000), the effects of neuropeptides on microglial-derived chemokines are not known. Since VIP and PACAP function as important immunomodulators in the periphery (Pozo et al., 2000; Ganea and Delgado, 2001a, 2001b; Gomariz et al., 2001), we investigated their potential role in microglial chemokine production. In the present study, we focused on the inflammatory CXC (KC and MIP-2) and CC (MIP-1α, MIP-1β, MCP-1, and RANTES) chemokines and investigated the involvement of specific receptors, intracellular signal pathways, and nuclear transactivating factors.

VIP and PACAP inhibit inflammatory chemokine production in LPS- and cytokine-activated microglia. The inhibitory effect is dose-dependent within a wide range of neuropeptide concentrations (10^{-10}-10^{-6} M), with the maximum effect at 10^{-8} M. This is the dose range in which VIP and PACAP modulate several other immunological functions, such as inhibition of macrophage-derived cytokine and chemokine production, and inhibition of TNFα production by LPS-stimulated microglia (Delgado et al., 1999b; Kim et al., 2000; Pozo et al., 2000; Delgado and Ganea, 2001a). VIP and PACAP act through three specific receptors, i.e., VPAC1, VPAC2, and PAC1 (Harmar et al., 1998). Microglia have been shown to express VIP/PACAP-binding sites (Kim et al., 2000), and our data demonstrate that, similar to rat microglia (Kim et al., 2000), mouse primary microglia and the microglia cell lines EOC13 and BV2 express VPAC1 and PAC1, but not VPAC2, for at least 12 h following LPS stimulation. Our agonist studies suggest that VPAC1 mediates the inhibitory effect on CXC and CC chemokine production. The role of VPAC1 as the unique mediator in the effect on chemokine production is also supported by the fact that a VPAC1 antagonist, but not $PACAP_{6-38}$, an antagonist specific for PAC1 and to a lesser degree for VPAC2, reverses the inhibitory effect of VIP/PACAP.

VPAC1 is coupled primarily to the adenylate cyclase system (Harmar et al., 1998), and LPS-induced production of some of the chemokines (i.e., MCP-1, MIP-1α, MIP-1β, MCP-3, MIP-2, RANTES) is indeed inhibited by agents that increase intracellular cAMP levels (Martin and Dorf, 1991; Hasko et al., 1998; Kimata et al., 1998; Shenkar and Abraham, 1999; Ammit et al., 2000; Kondo et al., 2000). In the present study, forskolin, a cAMP-inducing agent, and db-cAMP, a cAMP analog, inhibited chemokine production. In addition, H89, a potent and selective PKA inhibitor, reversed the inhibitory effect of VIP/PACAP, supporting the involvement of the cAMP/PKA pathway.

Whereas posttranscriptional, translational, and posttranslational mechanisms play important roles, transcription appears to be the primary regulatory site for chemokine synthesis. The present study indicates

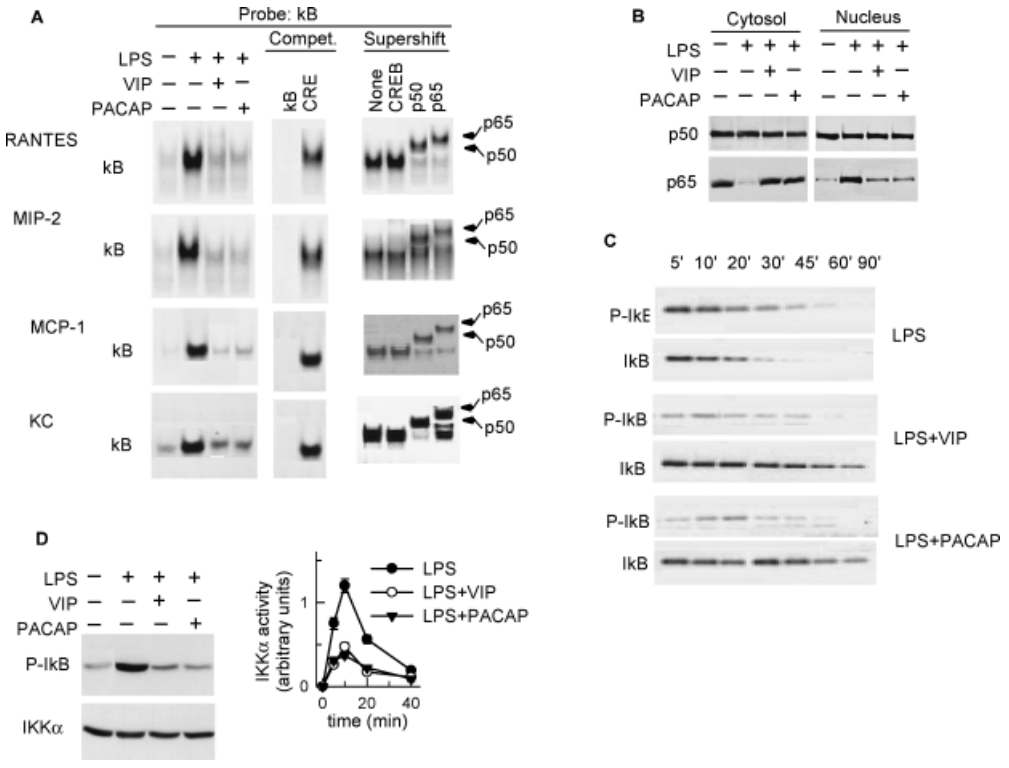

Fig. 7. VIP and PACAP prevent LPS-induced IkB degradation and subsequent NF-kB nuclear translocation and DNA binding. **A:** VIP and PACAP inhibit NF-kB DNA binding. Nuclear extracts were prepared from BV2 cells incubated for 2 h with LPS (100 ng/ml) in the presence or absence of VIP or PACAP (10^{-8} M). NF-kB binding was assessed by EMSA using radiolabeled oligonucleotides containing the murine NF-kB site from the RANTES, MIP-1α, MCP-1, and KC promoters. Middle panel: Specificity was assessed by the addition of 50-fold excess unlabeled homologous (NF-kB), or nonhomologous (CRE) oligonucleotides. Right panel: Identification of the proteins bound to the NF-kB site, by supershift assays. Nuclear extracts were incubated with polyclonal antibodies against CREB, p65, or p50 for 20 min before adding the radiolabeled probe. Similar results were observed in three independent experiments. **B:** VIP and PACAP inhibit LPS-induced p65 translocation. BV2 cells were incubated with medium alone (unstimulated) or stimulated with LPS (100 ng/ml) in the presence or absence of VIP or PACAP (10^{-8} M). After 1-h incubation, cytosolic and nuclear proteins were extracted, and Western blot analysis was performed for p50 and p65 in both cytoplasmic and nuclear extracts. One representative experiment of three is shown. **C:** VIP and PACAP prevent LPS-induced IkBα phosphorylation and subsequent degradation. BV2 cells were stimulated with LPS (100 ng/ml) in the presence or absence of VIP or PACAP (10^{-8} M). The cytosolic amounts of IkBα and phosphorylated-IkBα at different time points were determined by Western blot. One representative experiment of three is shown. **D:** VIP and PACAP inhibit IKKα activity. BV2 cells were stimulated with LPS (100 ng/ml) in the presence or absence of VIP or PACAP (10^{-8} M) for different time periods (10 min for blots in top panel). IKKα activity was assayed in an in vitro kinase assay. Lower panel: IKKα activity is expressed as arbitrary densitometric units. Data represent the mean ± SD of three independent assays. As control, the amounts of IKKα were determined by immunoblotting with anti-IKKα Ab (top panel).

that VIP and PACAP reduce the chemokine mRNA levels. An obvious question is how VIP and PACAP regulate such a wide spectrum of chemokines? A possible answer resides in the fact that transcription of most chemokines depends on activation of the pleiotropic transcription factor NF-kB (Freter et al., 1992, 1995, 1996; Grove and Plumb, 1993; Nelson et al., 1993; Widmer et al., 1993; Ohmori et al., 1995). Cytoplasmic NF-kB, consisting mostly of p50/p65 heterodimers, is complexed to the IkB inhibitor in unstimulated cells; stimuli such as LPS and proinflammatory cytokines induce the phosphorylation and degradation of IkB, followed by the release and subsequent nuclear translocation of NFkB, which then binds to regulatory sequences in a variety of target genes (Baldwin, 1996; Karin and Ben-Neriah, 2000). The present study indicates that, in the microglial cell line BV2, VIP and PACAP inhibit LPS-induced p65 nuclear translocation and its subsequent binding to the kB motifs found in the promoters of KC, RANTES, MCP-1, and MIP-1α. The inhibition of p65 translocation by VIP/PACAP is mediated through the stabilization of IkBα by inhibiting IkBα phosphorylation and its subsequent degradation. This is accomplished through an inhibitory effect on IKKα. All these effects are mediated through VPAC1 and cAMP. A similar inhibitory effect on NFkB binding to the promoters of proinflammatory mediators was described for macrophages and monocytes (Delgado M et al., 1998, 1999a, 1999b; Delgado and Ganea, 1999). However, whereas VIP/PACAP inhibition of NF-kB binding in activated BV2 cells is entirely cAMP-dependent, in macrophages and monocytes, the inhibition of NFkB transactivating activity is mediated through both a cAMP-dependent and -independent mechanisms (Delgado and Ganea, 2001b). These findings indicate that, even if microglia and macrophages belong to the same lineage and share many functions, differences exist in the VIP/

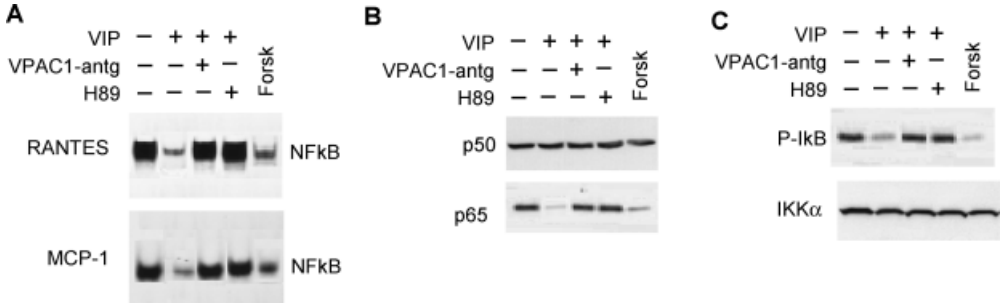

Fig. 8. Receptors and intracellular pathways involved in the VIP and PACAP regulation of NFkB nuclear translocation and IKK activity. BV2 cells were activated with LPS (100 ng/ml) in the absence (lanes 1) or presence of VIP (10^{-8} M, lanes 2), or forskolin (10^{-6} M, lanes 5). VPAC1 antagonist (10^{-7} M, lanes 3) or H89 (100 ng/ml, lanes 4) were added simultaneously with VIP (10^{-8} M). **A:** NF-kB binding was analyzed 1 h after stimulation by EMSA as described in Figure 7. One representative experiment of three is shown. **B:** After 1-h incubation, nuclear proteins were extracted, and Western blot analysis was performed for p50 and p65 in nuclear extracts. One representative experiment of four is shown. **C:** IKKα activity (20 min after stimulation) was analyzed with IkBα as substrate by using an in vitro kinase assay. One representative experiment of three is shown.

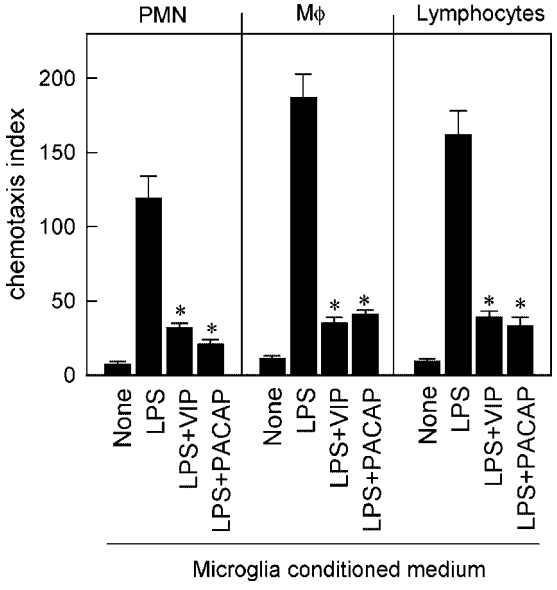

Fig. 9. VIP and PACAP inhibit microglia-induced chemotactic activity for peritoneal leukocytes. Primary microglia were stimulated with LPS in the presence or absence of VIP or PACAP (10^{-8} M). Supernatants collected 24 h later were seeded in lower wells of 48-well chemotaxis microchamber, and chemotaxis of freshly isolated murine peritoneal cells was assayed at 37°C for 90 min as described in text. At the end of the incubation period, the filters were stained, and the number of the different peritoneal cell populations that migrated were evaluated (chemotaxis index). Complete medium instead culture supernatants was used as basal control (none). Results are expressed as mean ± SD of triplicate determinations of one representative experiment of four.

PACAP transduction pathways for the regulation of proinflammatory factors.

Of obvious biological significance is the fact that VIP and PACAP reduce the chemotactic activity generated by activated microglia for peripheral leukocytes, i.e., neutrophils, macrophages, and lymphocytes. The reduction in chemokine production is in good correlation with the decrease in chemotactic activity, suggesting that the effect of VIP/PACAP on microglia-derived chemokines has significant physiological consequences. Reduction in the number and activation of infiltrating leukocytes represents an important factor in the control of inflammation in the CNS.

These data lead us to propose a model for the interactions between cells in the CNS during a normal response to infectious agents or to traumatic injury. Although the initial step of antigen uptake and presentation is not entirely clear, microglia, and to a certain extent astrocytes, can function as antigen-presenting cells, upregulating the expression of MHC class II, CD40, and B7 molecules. Presentation of the antigen, possibly to patrolling T-cells, contributes to further activation of glial cells, characterized by secretion of chemokines, cytokines, nitric oxide, and oxygen radicals. All these proinflammatory factors attract and further activate immune cells in the CNS and contribute to the direct destruction of the invading pathogen. However, an active proinflammatory response in the CNS can also damage and kill neighboring cells, including neurons. It would make sense that, faced with a nearby inflammatory response, neurons will try to protect themselves by secreting anti-inflammatory factors. It is interesting that neuronal injury leads to a significant increase in neuropeptide message, and further that most neuropeptides are indeed anti-inflammatory. In this context, VIP and/or PACAP released by neurons during an inflammatory response could serve as neuronal survival factors by limiting the inflammatory process.

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