

Vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide inhibit the production of inflammatory mediators by activated microglia

Mario Delgado,^{*,†} Javier Leceta,[†] and Doina Ganea^{*}

^{*}Department of Biological Sciences, Rutgers University, Newark, New Jersey; and [†]Departamento Biología Celular, Facultad de Biología, Universidad Complutense, Madrid, Spain

Microglia play a central role in the regulation of immune and inflammatory activities, as well as tissue remodeling in the central nervous system. However, activation of microglia is a histopathological hallmark of several neurodegenerative diseases. Pathological microglial activation is believed to contribute to progressive damage in neurodegenerative diseases through the release of proinflammatory and/or cytotoxic factors, including tumor necrosis factor α (TNF- α), interleukin (IL)-1 β , IL-6, IL-12, and nitric oxide (NO). Hence, it is important to unravel mechanisms regulating microglia activation of inflamed brain parenchyma to provide insights into efficient therapeutic intervention. This study examines the role of two anti-inflammatory neuropeptides, the vasoactive intestinal peptide (VIP) and the pituitary adenylate cyclase-activating polypeptide (PACAP) on the production of various proinflammatory factors by endotoxin-stimulated microglia. VIP and PACAP inhibit TNF- α , IL-1 β , IL-6, and NO production by lipopolysaccharide (LPS)-activated microglia. The specific type 1 VIP receptor mediates the inhibitory effect of VIP/PACAP, and cyclic adenosine monophosphate is the major, second messenger involved. VIP and PACAP regulate the production of these proinflammatory factors at a transcriptional level by inhibiting p65 nuclear translocation and nuclear factor- κ B-DNA binding. This effect is mediated, as neuropeptides stabilize the inhibitor I κ B by inhibiting LPS-induced I κ B-kinase activity. Therefore, the inhibitory effects on the production of proinflammatory mediators define VIP and PACAP as “microglia-deactivating factors” with significant, therapeutic potential for inflammatory/degenerative brain disorders. *J. Leukoc. Biol.* 73: 155–164; 2003.

Key Words: neuroimmunology · neuropeptides · cytokines · inflammation · central nervous system

INTRODUCTION

Microglia, the ontogenetic and functional equivalents of mononuclear phagocytes in somatic tissues [1], are widely recog-

nized as cells that play a central role in the regulation of immune and inflammatory activities, as well as tissue remodeling in the central nervous system (CNS). Microglia serve as antigen-presenting cells of the CNS, control the proliferation of astrocytes, produce cytokines and other soluble factors associated with an immunologic response, and remove tissue debris during development and following brain trauma [2, 3]. However, these cells may also play a pathogenetic role in a variety of CNS diseases, such as multiple sclerosis [4], Alzheimer's disease [5], Parkinson's disease [6], and AIDS dementia [1, 7–9]. Although the precise mechanism by which microglia mediate neuronal cell injury is incompletely understood, it has been proposed that in response to proinflammatory cytokines or antigens such as lipopolysaccharides (LPS), activated microglia secrete inflammatory mediators such as nitric oxide (NO) and proinflammatory cytokines, including tumor necrosis factor α (TNF- α), interleukin (IL)-6, and IL-1 β , which contribute to pathophysiological changes associated with several neuroimmunologic disorders [10–19]. Many studies have highlighted the strict link between production of these factors and the nature and intensity of the host inflammatory response in the CNS. Secretion of proinflammatory products is followed later by the production of anti-inflammatory cytokines such as IL-10 and tumor growth factor- β . As the intensity and duration of an inflammatory process depend on the local balance between pro- and anti-inflammatory factors, a number of regulatory molecules termed microglia-deactivating factors have been the focus of considerable research.

Vasoactive intestinal peptide (VIP) and the structurally related peptide, the pituitary adenylate cyclase-activating polypeptide (PACAP), are two neuropeptides that elicit a broad spectrum of biological functions, including actions on natural and acquired immunity [20–23]. Although VIP and PACAP affect a variety of immune functions, their primary, immunomodulatory function is anti-inflammatory in nature. VIP and PACAP have been shown to inhibit cytokine production and proliferation in T cells and to inhibit several macrophage functions, including phagocytosis, respiratory burst, and chemotaxis (reviewed in refs. [20–23]), as well as LPS-induced

Correspondence: Mario Delgado, Department Cell Biology, School of Biology, Complutense University, Madrid 28040, Spain. E-mail: mariodm@bio.ucm.es

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IL-6, TNF- α , IL-12, NO, and chemokine production [24–31]. In agreement with their anti-inflammatory role, VIP/PACAP were reported to protect mice from lethal endotoxemia, presumably by down-regulating endogenous, proinflammatory, macrophage-derived mediators [32].

As microglia-derived inflammatory factors are involved in controlling the nature and magnitude of the inflammatory response in the CNS, in this study, we examine the effects of both neuropeptides on TNF- α , IL-6, IL-1 β , and NO production in activated microglia. We also investigate the molecular mechanisms involved, including the specific receptors, the intracellular signal pathways, and the nuclear-transactivating factors that mediate the effect of VIP/PACAP. This study may further clarify the role played by VIP and PACAP in the attenuation of the inflammatory response.

MATERIALS AND METHODS

Reagents

Synthetic VIP and PACAP38 were purchased from Calbiochem-Novabiochem (Laufelfingen, Switzerland). The PACAP receptor (PAC1)/type 2 VIP receptor (VPAC2) antagonist (PACAP_{6–38}) was obtained from Peninsula Laboratories (Belmont, CA). The VPAC1 antagonist (Ac-His¹, D-Phe², K¹⁵, R¹⁶, L²⁷) VIP (3–7)-growth-hormone-releasing factor (GRF) (8–27) and the VPAC1 agonist (K¹⁵, R¹⁶, L²⁷) 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-(Glu⁸, Lys¹², Nle¹⁷, Ala¹⁹, Asp²⁵, Leu²⁶, Lys^{27,28}, Gly^{29,30}, Thr³¹)-VIP cyclo (21–25) was a generous gift from Drs. Ann Welton and David R. Bolin (Hoffmann-La Roche, Inc., Nutley, NJ). The synthetic PAC1 agonist maxadilan was a generous gift from Dr. Ethan A. Lerner (Massachusetts General Hospital, Charlestown). Murine recombinant (mr)TNF- α , IL-6, and IL-1 β and capture and biotinylated antibodies against mIL-6 were purchased from PharMingen (San Diego, CA). LPS (from *Escherichia coli* 055:B5), protease inhibitors, dibutyl-cyclic adenosine monophosphate (db-cAMP), and forskolin were purchased from Sigma Chemical Co. (St. Louis, MO), and N-[2-(p-bromocinnamyl-amino)ethyl]-5-iso-quinolinesulfonamide (H89) was from ICN Pharmaceuticals Inc. (Costa Mesa, CA). α IKB α (1–317) and antibodies against p65, p50, IKB α , phosphorylated- α IKB α , IKB-kinase (IKK α), and cAMP response element-binding protein (CREB) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell cultures

Microglial cell cultures were prepared as described previously [33]. Briefly, cerebral cortical cells from 1-day-old BALB/c mice were dissociated after a 30-min trypsinization (0.25%) and were plated in 75-cm² Falcon culture flasks in Dulbecco's modified Eagle's medium high-glucose formula (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% heat-inactivated fetal calf serum (Gibco-BRL, Grand Island, NY), containing 10 mM HEPES buffer, 1 mM pyruvate, 0.1 M nonessential amino acids, 2 mM glutamine, 50 mM 2-mercaptoethanol, 100 U/ml penicillin, and 10 μ g/ml streptomycin (complete medium). The medium was replenished 1 and 4 days after plating, and on day 8 of culture, plates were shaken for 20 min at a speed of 200 rpm in an orbital shaker to remove oligodendrocytes. On day 12 of culture, plates were shaken again for 2 h at a speed of 180–200 rpm. Harvested cells were filtered through a 20- μ m nylon mesh, plated in a 60-mm petri dish, and incubated for 15 min at 37°C. After extensive washing with culture medium, adherent cells (microglia) were collected with a rubber policeman and were centrifuged (1000 rpm, 10 min). Purified microglial cell cultures were comprised of a cell population in which >98% stained positively with MAC-1 antibodies (Boehringer Mannheim Biochemicals, Indianapolis, IN), and <2% stained positively with antibodies specific to the astrocyte marker glial fibrillary acid protein (Sigma Chemical Co.).

Microglia monolayers were incubated with complete medium and stimulated with 500 ng/ml LPS in the presence or absence of VIP or PACAP38 (from

10⁻¹² to 10⁻⁶ M) at 37°C in a humidified incubator with 5% CO₂. Cell-free supernatants were harvested at the designated time points and kept frozen (-20°C) until cytokine and nitrite determination.

NO and cytokine assays

The activity of IL-1 β was determined by bioassay using D-10 cells by means of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide colorimetric assay [34]. TNF- α activity was measured by cytotoxicity to L929 cells as described previously [35]. The content of IL-6 in the culture supernatants was determined by specific sandwich enzyme-linked immunosorbent assays, as described previously [24]. The production of NO was assessed as the accumulation of nitrite in the culture supernatants using the colorimetric reaction with the Griess reaction as described previously [27].

Electrophoretic mobility-shift assay (EMSA)

Nuclear extracts were prepared by the mini-extraction procedure as described previously [27]. Briefly, microglia cells were cultured at a density of 10⁷ cells in six-well plates, stimulated as described above, washed twice with ice-cold phosphate-buffered saline/0.1% bovine serum albumin, and harvested from the dishes. The cell pellets were homogenized with 0.4 ml buffer A [10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 10 μ g/ml pepstatin, and 1 mM Na₃N]. After 15 min on ice, Nonidet P-40 was added to a final 0.5% concentration, the tubes were gently vortexed for 15 s, and nuclei were sedimented and separated from cytosol by centrifugation at 12,000 g for 40 s. Pelleted nuclei were washed once with 0.2 ml ice-cold buffer A, and the soluble nuclear proteins were released by adding 0.1 ml buffer C (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 25% glycerol, 1 mM DTT, 0.5 mM PMSF, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 10 μ g/ml pepstatin, and 1 mM Na₃N]. After incubation for 30 min on ice, followed by centrifugation for 10 min at 14,000 rpm at 4°C, the supernatants containing the nuclear proteins were harvested, the protein concentration was determined by the Bradford method, and aliquots were stored at -80°C for later use in EMSAs.

Double-stranded oligonucleotides (50 ng) corresponding to the nuclear factor (NF)- κ B sites from murine TNF- α (5'-CAAACAGGGGCTTTCCCTC-CTC-3') [36], inducible NO synthase (iNOS; 5'-CCAACTGGGACTCTC-CCTTTGGGAACA-3') [37], IL-6 (5'-ATGTGGGATTTTCCCATGAG-3') [38], and IL-1 β (5'-AGAGCTGAATAATTCCTCCAAA-3') [39] were end-labeled with [γ -³²P]adenosine 5'-triphosphate (ATP) by using T4 polynucleotide kinase. For EMSAs with microglia nuclear extracts, 20,000–50,000 cpm double-stranded oligonucleotides corresponding to ~0.5 ng were used for each reaction. The binding-reaction mixtures (15 μ l) were set up containing 0.5–1 ng DNA probe, 5 μ g nuclear extract, 2 μ g 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 another 20 min at room temperature. Samples were loaded onto 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, drying under vacuum at 80°C, and autoradiography. In competition and antibody-supershift experiments, the nuclear extracts were incubated for 15 min at room temperature with the specific antibody (1 μ g) or competing cold oligonucleotide (50-fold excess) before the addition of the labeled probe.

mRNA analysis

Murine, primary microglia cells were cultured at a concentration of 2 x 10⁶ cells/ml in 100 mm tissue-culture dishes and were stimulated with LPS (500 ng/ml) in the presence or absence of VIP (10⁻⁸ M) or PACAP (10⁻⁸ M) 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 (Biotech, Houston, TX) as recommended by the manufacturer. RNase protection assays (RPA) were performed on 2.5–5 μ g RNA using the Riboquant MultiProbe RNase protection assay system (PharMingen) following the manufacturer's instructions. TNF- α and iNOS mRNA levels were determined by Northern blot analysis according to standard methods [26, 27]. The membranes were exposed to X-ray films, and signal quantitation was performed in a PhosphorImager SI (Molecular Dynamics, Sunnyvale, CA).

Western blotting

For Western blot analysis, whole-cell lysates, cytoplasmic fraction, or nuclear extract (see above) containing 20–30 μ g protein were subjected to reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 12.5%). After electrophoresis, the gel was electroblotted in Tris-glycine buffer containing 40% methanol onto a reinforced nitrocellulose membrane (Schleicher-Schuell, Keene, NH). The membrane was blocked with Tris-buffered saline/Tween 20 (TBS-T) buffer (10 mM Tris, pH 8.0, 150 mM NaCl, 0.05% Tween 20) containing 5% milk powder for 1 h at room temperature and was then incubated with primary antibodies at dilutions ranging from 1:500 to 1:2000, rabbit anti-mouse immunoglobulin G (IgG) against I κ B α , IKK α , NF- κ B p50, or NF- κ B p65, or with mouse IgG against phosphorylated I κ B α in TBS-T containing 1% milk powder for 2 h at room temperature. The membrane was washed with TBS-T and incubated with secondary antibody, peroxidase-conjugated goat anti-rabbit IgG or rat anti-mouse IgG at a 1:5000 dilution for 1 h at room temperature. After washing three times in TBS-T for 5 min each and once in TBS for 5 min, the membrane was drained briefly and subjected to the enhanced chemiluminescence detection system (Amersham, Little Chalfont, UK). The X-ray films were exposed for 5–20 min.

In vitro kinase assay

In vitro IKK α kinase assay was performed as described previously [40]. Briefly, whole-cell lysates were prepared by lysing 2×10^6 cells in 200 μ l lysis buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 2 mM EGTA, 50 mM glycerol phosphate, 1% Triton X-100, 10% glycerol, 1 mM DTT, 2 μ g/ml leupeptin, 5 μ g/ml aprotinin, 1 mM PMSF, 5 mM NaF, 10 mM p-nitrophenyl phosphate, and 1 mM Na_3VO_4). The cell lysates were kept on ice and vigorously vortexed every 5 min for 20 min. The lysates were cleared by centrifugation at 13,000 g for 3 min, and the supernatants were stored at -80°C . Endogenous IKK α was immunoprecipitated from cell lysates (150–250 μ g/sample) by incubation with 0.5 μ g anti-IKK α 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 . The beads were extensively washed with lysis buffer, twice with LiCl buffer, and twice with kinase buffer (20 mM MOPS, pH 7.6, 2 mM EGTA, 10 mM MgCl_2 , 1 mM DTT, 0.1% Triton X-100, 1 mM p-nitrophenyl phosphate, and 1 mM Na_3VO_4). The pelleted beads were resuspended in 30 μ l kinase buffer with 15 μ M ATP and 10 μ Ci [γ - ^{32}P]ATP (3000 Ci/mmol) containing 5 μ g rI κ B α . The kinase reaction was performed at 30°C for 30 min and stopped by the addition of 15 μ l 2 $\times$ SDS sample buffer. Following boiling for 5 min, the samples were subjected to SDS-PAGE (9%).

Proteins were transferred onto a nitrocellulose membrane (Schleicher-Schuell) followed by autoradiography. The IKK α kinase activity was determined by the incorporation of ^{32}P into its substrate and quantitated by phosphoimaging. Expression of the IKK α was verified by immunoblotting aliquots of cell lysates as described above.

RESULTS

VIP and PACAP inhibit LPS-induced production of proinflammatory factors by microglia

To determine the effect of VIP and PACAP on endotoxin-induced chemokine production in the CNS, murine primary microglia cells were activated with LPS, a potent activator of cells of the monocyte/macrophage cell lineage, in the absence or presence of various doses of VIP or PACAP, and the amounts of different proinflammatory factors (TNF- α , IL-6, IL-1 β , and NO) released in the culture supernatants were assayed at different time periods. Unstimulated microglia produce very low amounts of any cytokine (Fig. 1A). However, LPS stimulation of microglia cultures resulted in a time-dependent increase in the production of TNF- α , IL-6, IL-1 β , and nitrite, reaching peak levels between 8 and 16 h for TNF- α , IL-6, and IL-1 β and between 16 and 48 h for nitrite production (Fig. 1A). VIP and PACAP inhibit, in a dose- and time-dependent manner, the production of all proinflammatory factors by LPS-stimulated microglia (Fig. 1). Cytokine production was significantly inhibited as early as 2 h, with maximum inhibitory effect after 8–16 h of culture (Fig. 1A). However, VIP/PACAP inhibition of NO production was exerted slightly later (between 16 and 48 h; Fig. 1A). The dose-response curves were similar for VIP and PACAP, showing maximal effects around 10^{-8} M (Fig. 1B). The inhibitory effect of both neuropeptides was observed over a wide range of LPS concentra-

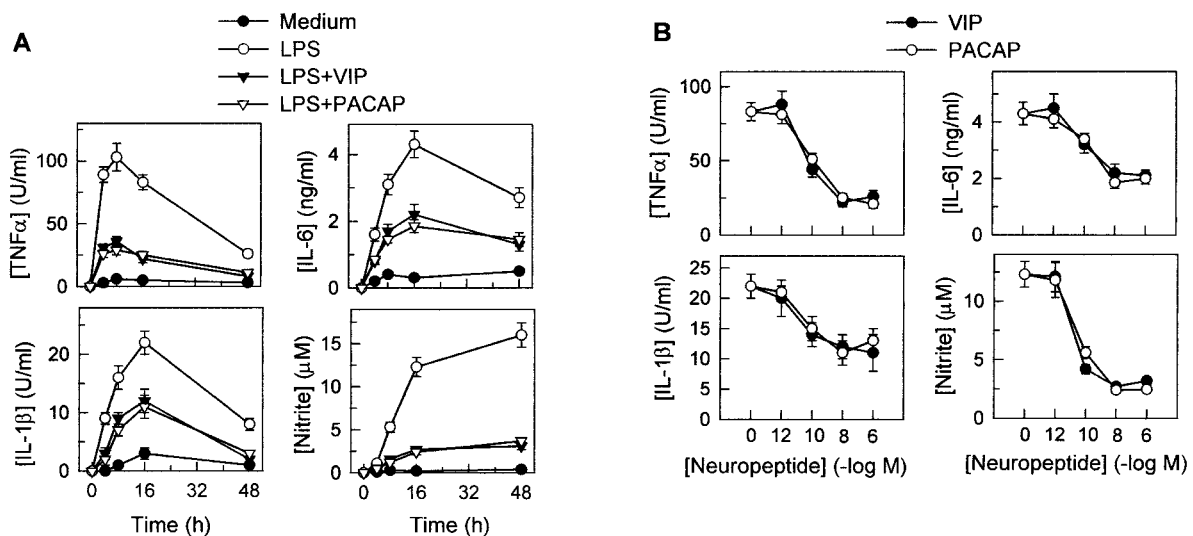


Fig. 1. VIP and PACAP inhibit LPS-induced proinflammatory cytokine production by activated microglia. (A) Time-dependent inhibition. Primary microglia (2×10^5 cells/ml) were stimulated with LPS (500 ng/ml) in the absence or presence of 10^{-8} M VIP or PACAP, and supernatants harvested at different time points were assayed for cytokine content as described in Materials and Methods. (B) Dose-dependent inhibition. Primary microglia were stimulated with LPS (500 ng/ml) and treated with various concentrations of VIP or PACAP for 16 h. The cytokine contents in the culture supernatants were determined as described in Materials and Methods. Each result is the mean $\pm$ SD of four separate experiments performed in duplicate.

tions, showing maximal effect at 10–500 ng/ml LPS (data not shown).

The inhibitory effects were not the result of a decreased number of microglia 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).

As the highest inhibition of production of most inflammatory factors was obtained with microglia cultures stimulated with 500 ng/ml LPS, at a neuropeptide concentration of 10^{-8} M, after 16 h of culture, we use these conditions in the rest of the experiments.

Inhibition of cytokine production by VIP and PACAP is mediated through VPAC1

Next, we investigated whether the inhibitory effect of VIP/PACAP could be related to occupancy of specific receptors. VIP and PACAP act through a family of receptors consisting of VPAC1, VPAC2, and PAC1 [41]. Reverse transcriptase-polymerase chain reaction analysis indicates that murine primary microglia express PAC1 and VPAC1 mRNA [42]. In contrast, VPAC2 mRNA was not expressed even following LPS activation. A similar pattern of VIP/PACAP receptor expression was observed in rat primary microglia [43] and in the two murine microglia cell lines, EOC13 and BV2 [42]. To determine which of the VIP/PACAP receptors is involved in the inhibition of inflammatory cytokine production, we used specific receptor agonists and antagonists. The effects of a VPAC1 agonist [44], a VPAC2 agonist (Ro 25-1553) [45], and of maxadilan, a PAC1 agonist [46], were tested. The VPAC1 agonist but not the VPAC2 or the PAC1 agonist inhibits the release of TNF- α , IL-6, IL-1 β , and nitrite, with a potency similar to that of VIP/PACAP (Fig. 2A). In addition, we investigated the ability of PACAP_{6–38}, an antagonist specific for PAC1 and to a lesser degree for VPAC2 [47], and of a specific VPAC1 antagonist [48] to reverse the effects of VIP and PACAP. Increasing concentrations of the antagonists (10^{-8} – 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. 2B). In contrast, PACAP_{6–38} did not reverse the inhibitory effect (Fig. 2B). Together, these results indicate that neuropeptides exert their action primarily through VPAC1. In addition, the specificity of VIP and PACAP was also confirmed by the fact that other members of the VIP family, such as secretin and glucagon, as well as some VIP/PACAP fragments (VIP_{1–12}, VIP_{10–28}, and PACAP_{6–38}) did not show any significant effect on TNF- α , IL-6, IL-1 β , and NO production (data not shown).

Intracellular signal pathways involved in the inhibitory effect of VIP and PACAP on the production of inflammatory mediators

The VPAC1 is coupled primarily to the adenylate cyclase system [41], and LPS-induced production of some of these inflammatory factors (i.e., TNF- α , IL-1 β , IL-6, and NO) is indeed inhibited in activated microglia by agents that increase intracellular cAMP levels [49–53]. To determine whether intracellular cAMP is involved in the inhibitory effect of VIP/

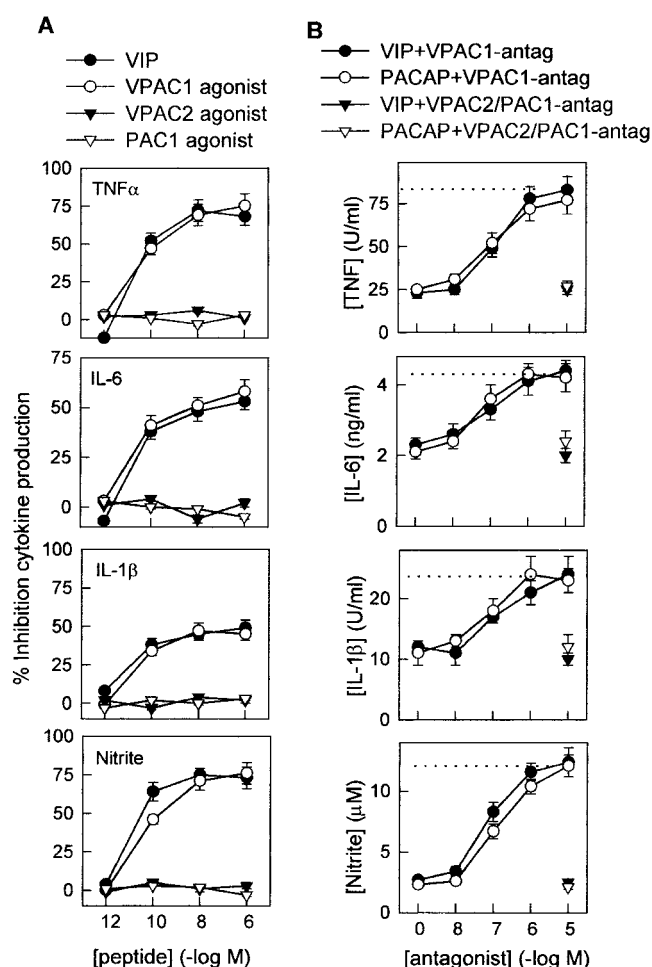


Fig. 2. The effect of VIP/PACAP is mediated through VPAC1. (A) Comparative effects of VIP/PACAP receptor agonists on cytokine production. Primary microglia were stimulated with LPS (500 ng/ml) in the presence or absence of different concentrations of VIP, maxadilan (a PAC1 agonist), Ro 25-1553 (a VPAC2 agonist), and (K¹⁵,R¹⁶,L²⁷) VIP (1–7)-GRF (8–27; a VPAC1 agonist). Supernatants collected 16 h later were assayed for cytokine production as described in Materials and Methods. Percentage inhibition was calculated by comparison with controls containing LPS alone. Each result is the mean $\pm$ SD of four experiments. Each sample was assayed in duplicate. (B) Effect of PAC1 and VPAC1 antagonists. Primary microglia were stimulated with LPS (500 ng/ml) and treated simultaneously with VIP or PACAP (10^{-8} M) and different concentrations of the VPAC1 antagonist (Ac-His¹,D-Phe²,K¹⁵,R¹⁶,L²⁷) VIP (3–7)-GRF (8–27) or the PAC1/VPAC2 antagonist (PACAP_{6–38}). Supernatants collected 16 h later were assayed for cytokine production as described in Materials and Methods. The VPAC1 antagonist (10^{-6} M) and PACAP_{6–38} (10^{-6} M) did not affect cytokine production in LPS-treated microglia. The dotted line represents control values from cultures incubated with LPS alone. Each result is the mean $\pm$ SD of four experiments performed in duplicate.

PACAP on cytokine production by activated microglia, we investigated the effects of calphostin C [a protein kinase C (PKC) inhibitor], H89 [a protein kinase A (PKA) inhibitor], forskolin (a strict cAMP-inducing agent), and db-cAMP (a cAMP analog). Forskolin and db-cAMP inhibit production of TNF- α , IL-6, IL-1 β , and nitrite in LPS-activated microglia in a similar way as that of VIP and PACAP (Fig. 3A). In addition, the involvement of cAMP is supported by the results obtained with the two protein kinase inhibitors. In contrast to calphostin C, H89 reverses the inhibitory effect of VIP/PACAP (Fig. 3B).

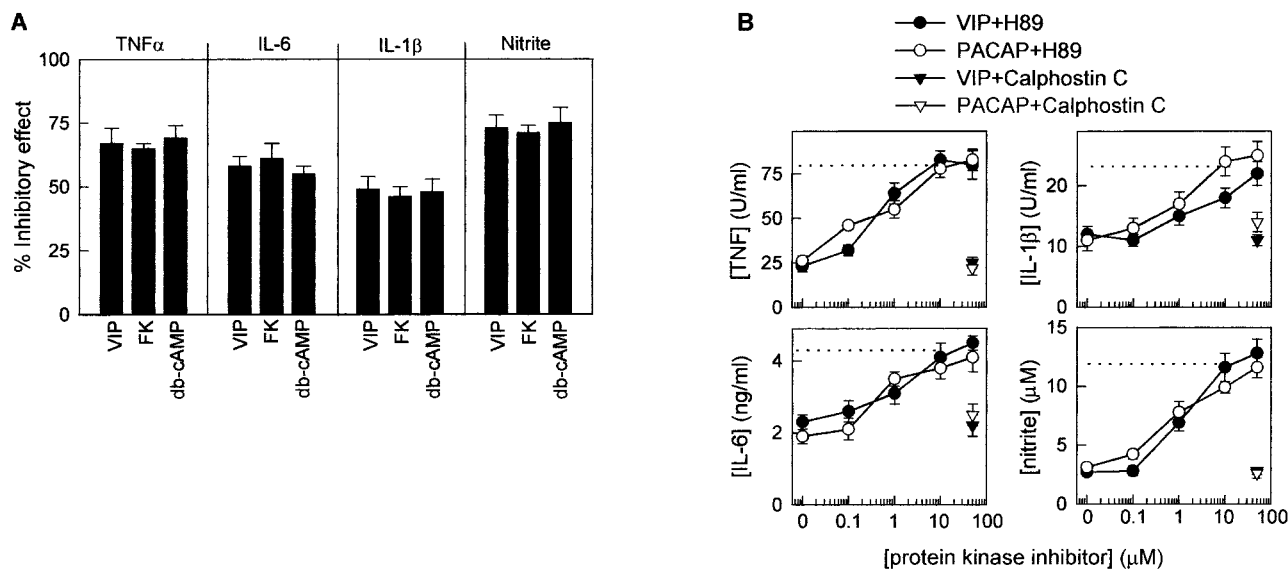


Fig. 3. Intracellular signal pathways involved in the VIP/PACAP inhibition of cytokine production. (A) Effect of cAMP-inducing agents. Primary microglia were stimulated with LPS (500 ng/ml) in the presence or absence of different concentrations of VIP, forskolin (FK), or db-cAMP. Supernatants collected 16 h later were assayed for cytokine production. Percentage inhibition was calculated by comparison with control cultures incubated with LPS alone. Each result is the mean $\pm$ 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 (500 ng/ml) and 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 16 h later were assayed for cytokine production. The dotted line represents control values from cultures incubated with LPS alone. Each result is the mean $\pm$ SD of four experiments performed in duplicate.

These results suggest that the inhibitory effect of VIP/PACAP is mediated at least partially through increases in intracellular cAMP.

VIP and PACAP regulate the production of several inflammatory mediators by microglia at the mRNA level

Having demonstrated that VIP and PACAP inhibit TNF- α , IL-6, IL-1 β , and NO production in murine microglia, we sought to determine whether this action occurs at a transcriptional level through the inhibition of mRNA expression. We stimulated primary microglial cells with LPS in the presence or absence of 10^{-8} M VIP or PACAP for 2, 6, 12, 18, and 24 h, and total RNA was prepared and subjected to RPA and Northern blot analysis. Although no or very little cytokine mRNA is detectable in unstimulated cells (Fig. 4), progressively increased levels of TNF- α , IL-6, and IL-1 β mRNA are present in LPS-stimulated cells (up to 3–12 h; Fig. 4). In contrast to neurons and endothelial cells that express a constitutive NOS, microglia express an iNOS responsible for the prolonged, high-output production of NO. An increased expression of iNOS mRNA was observed in LPS-stimulated microglia (up to 12–24 h; Fig. 4). In addition, we observed an increased mRNA expression of other proinflammatory cytokines such as IL-12p40, IL-1 α , IL-18, and macrophage inflammatory factor (MIF) after activation of microglia with endotoxin (Fig. 4). At all time points, VIP and PACAP significantly inhibited the levels of TNF- α , IL-6, IL-1 β , and iNOS mRNA (Fig. 4). Similarly, both neuropeptides inhibited LPS-induced mRNA expression of proinflammatory cytokines, such as IL-12p40, IL-1 α , IL-18, and MIF (Fig. 4). In contrast, VIP and PACAP stimulated steady-state mRNA levels of some anti-inflam-

tory cytokines such as IL-10 and IL-1Ra (Fig. 4). These results indicate that both neuropeptides regulate the production of several inflammatory mediators at the mRNA level.

VIP and PACAP prevent NF- κ B binding to the promoters of inflammatory cytokines and inhibit the subsequent NF- κ B-dependent gene activation

Although the promoters of most of proinflammatory mediators contain complex arrays of transactivating binding sites, NF- κ B appears to be essential for maximal cytokine transcription after LPS stimulation [54]. In macrophages/monocytes, VIP and PACAP inhibit production of several proinflammatory cytokines by down-regulating NF- κ B binding and transactivation [26, 27, 31, 40]. To investigate whether VIP/PACAP affect NF- κ B binding, we used EMSAs. Stimulation of microglia with LPS led to an increase in NF- κ B binding to TNF- α , IL-6, IL-1 β , and iNOS promoters compared with unstimulated cells; in all cases, treatment with VIP and PACAP significantly inhibited the binding (Fig. 5A, left panels). The specificity of the NF- κ B binding was evident by the complete displacement of the NF- κ B/DNA-binding complexes in the presence of a 50-fold excess of unlabeled, homologous oligonucleotides in the competition reactions (Fig. 5A, middle panels). In contrast, a 50-fold excess of unlabeled, nonhomologous oligonucleotides had no effects on this DNA-binding activity (Fig. 5A, middle panels). Antibody supershift experiments were performed to determine the composition of the NF- κ B-binding factors. Addition of monospecific anti-p50 or anti-p65 Ab to the binding reaction resulted in a marked reduction in the intensity of the NF- κ B band and led to the appearance of slow, migrating

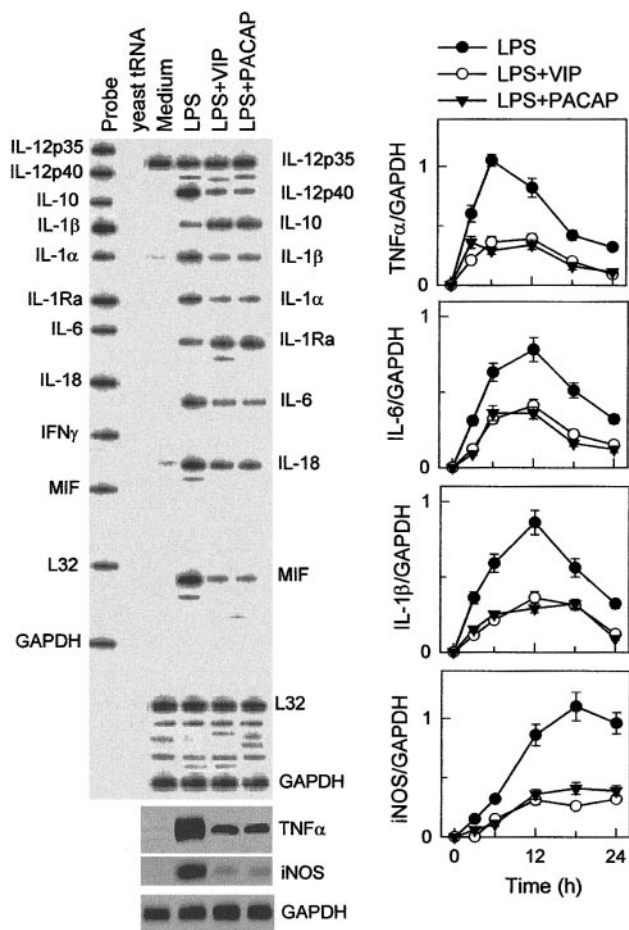


Fig. 4. VIP and PACAP regulate cytokine mRNA expression by LPS-stimulated microglia. Primary microglia were stimulated with LPS (500 ng/ml) in the presence or absence 10^{-8} M VIP or PACAP. At different time points (6 h for TNF- α blot, 12 h for RPA blot, 18 h for iNOS blot, left panel), the cells were lysed and subjected to RPA or TNF- α and iNOS Northern blot analysis. Results (mean $\pm$ SD) of two RPA and Northern blot assays performed on two samples) are expressed in arbitrary densitometric units normalized for the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in each sample.

bands, indicating that the NF- κ B-binding complex is composed primarily of p50/p65 heterodimers (Fig. 5A, right panel).

The primary level of control for NF- κ B is mediated through its interaction with the inhibitor I κ B. VIP and PACAP could inhibit NF- κ B activity by blocking LPS-induced I κ B degradation and subsequent NF- κ B 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 the level of cytoplasmic p65 and an increase in nuclear p65 levels (Fig. 5B). VIP and PACAP abolished the LPS-induced change in p65 levels (Fig. 5B), which indicates an inhibition of p65 nuclear translocation by VIP and PACAP. Differences in p65 protein levels were not a result of differences in protein loading, as immunoblot analysis demonstrated similar levels of p50. To further elucidate if the VIP/PACAP block in p65 nuclear translocation is caused by interference with the LPS-induced degradation of I κ B, we examined the cytoplasmic I κ B α levels. As expected, we observed a time-dependent I κ B α

degradation, paralleled by an increase in I κ B α phosphorylation in LPS-stimulated cells (Fig. 5C). VIP or PACAP blocks the phosphorylation and subsequent degradation of I κ B α (Fig. 5C).

As the LPS activation of NF- κ B requires IKK-mediated phosphorylation of I κ B α , we determined whether VIP and PACAP inhibit IKK activity by using an *in vitro* kinase assay. Stimulation of microglia cells with LPS resulted in a time-dependent increase in IKK α activity, which was inhibited by VIP and PACAP (Fig. 5D). No differences in IKK α expression were observed (Fig. 5D).

These results demonstrate that VIP and PACAP inhibit NF- κ B nuclear translocation and subsequent DNA binding in LPS-activated microglial cells by blocking the IKK-mediated I κ B phosphorylation/degradation.

As the inhibitory effect of VIP on TNF- α , IL-6, IL-1 β , and NO production by activated microglia 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 κ B-binding complexes and IKK activity. The inhibitory activity of VIP on LPS-mediated NF- κ B binding, p65 nuclear translocation, and I κ B phosphorylation was completely reversed by the VPAC1 antagonist (Fig. 5E, lane 3) and by H89 (Fig. 5E, lane 4). These results suggest that the inhibition of NF- κ B binding by VIP is mediated through VPAC1 and is cAMP-dependent. This is supported by the fact that forskolin (a cAMP inducer) mimics the effect of VIP on NF- κ B binding, p65 nuclear translocation, and IKK activity (Fig. 5E, lane 5).

DISCUSSION

Under normal conditions, brain microglia are involved in immune surveillance and host defense against infectious agents. However, in response to brain injury, infection, or inflammation, microglia readily become activated in a way similar to peripheral-tissue macrophages, a process that includes differentiation and probably invasion and proliferation. Activation of microglia is a histopathological hallmark of several neurodegenerative diseases, including Alzheimer's and Parkinson's diseases, multiple sclerosis, and the AIDS dementia complex [1, 4–9]. Pathological microglial activation is believed to contribute to progressive damage in neurodegenerative diseases through the release of proinflammatory and/or cytotoxic factors, including TNF- α , IL-1 β , IL-6, IL-12, and NO. Hence, it is important to unravel mechanisms regulating microglia activation of inflamed brain parenchyma to provide insights into efficient therapeutic intervention. The present study shows that VIP and PACAP, two well-known anti-inflammatory neuropeptides, inhibit TNF- α , IL-1 β , IL-6, and NO production in activated microglia through the binding to the VPAC1 receptor and the subsequent activation of the cAMP/PKA pathway.

Synthesis of all these proinflammatory factors is controlled at several levels. Whereas post-transcriptional, translational, and post-translational mechanisms play important roles, gene transcription appears to be the primary regulatory site. The present study indicates that the inhibitory effect of VIP and PACAP on TNF- α , IL-1 β , IL-6, and NO production occurs through the

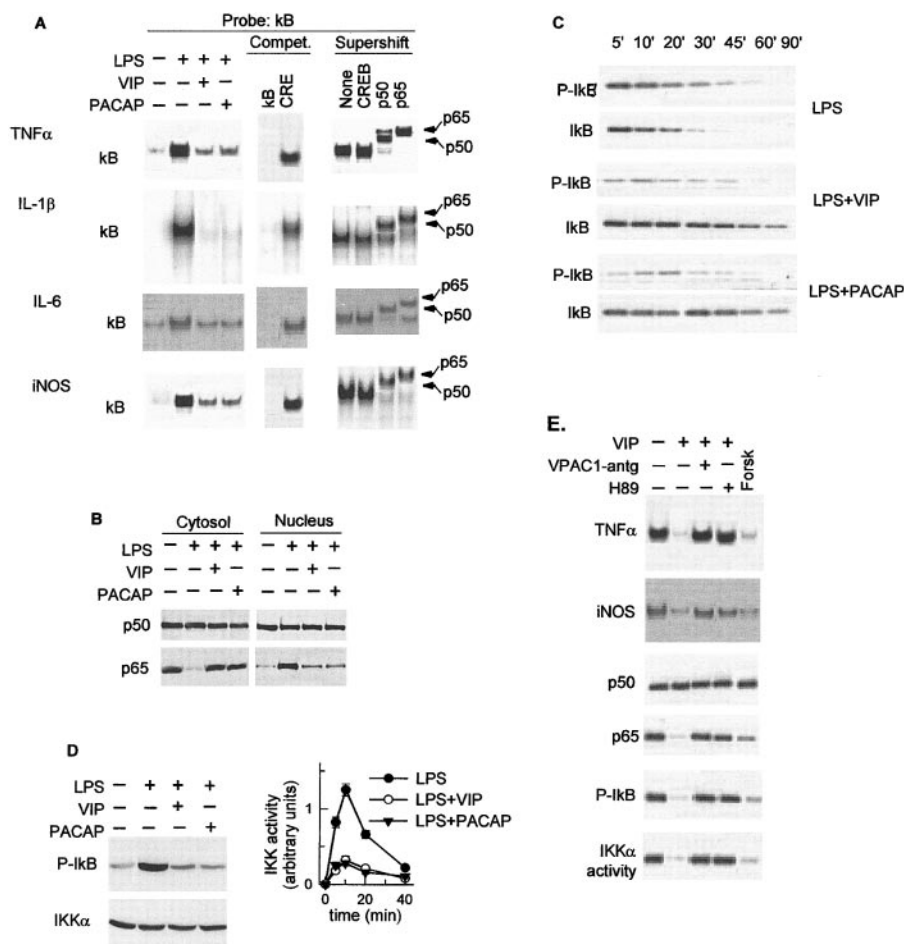


Fig. 5. VIP and PACAP prevent LPS-induced IkB degradation and subsequent NF-κB nuclear translocation and DNA binding. (A) VIP and PACAP inhibit NF-κB-DNA binding. Nuclear extracts were prepared from primary microglia incubated for 2 h with LPS (500 ng/ml) in the presence or absence of VIP or PACAP (10^{-8} M). NF-κB binding was assessed by EMSA using radiolabeled oligonucleotides containing the mNF-κB site from the TNF-α, IL-1β, IL-6, and iNOS promoters. (Middle panels) Specificity was assessed by the addition of 50-fold excess unlabeled, homologous (NF-κB; κB) or non-homologous (CRE) oligonucleotides. (Right panels) Identification of the proteins bound to the NF-κB site by supershift assays. Nuclear extracts were incubated with polyclonal antibodies against CREB, p50, or p65 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. Primary microglia were incubated with medium alone (unstimulated) or stimulated with LPS (500 ng/ml) in the presence or absence of VIP or PACAP (10^{-8} M). After 1 h incubation, cytosolic (Cytosol) and nuclear (Nucleus) proteins were extracted, and Western blot analysis was performed for p50 and p65 in cytoplasmic and nuclear extracts. One representative experiment of three is shown. (C) VIP and PACAP prevent LPS-induced IkBα phosphorylation and subsequent degradation. Primary microglia were stimulated with LPS (500 ng/ml) in the presence or absence of VIP or PACAP (10^{-8} M). The cytosolic amounts of IkBα (IkB) and phosphorylated IkBα (P-IkB) at different time points were determined by Western blot. One representative experiment of three is shown. (D) VIP and PACAP inhibit IKKα activity. Primary microglia were stim-

ulated with LPS (500 ng/ml) in the presence or absence of VIP or PACAP (10^{-8} M) for different time periods (10 min for blots in left panel). IKKα activity was assayed in an in vitro kinase assay. (Right panel) IKKα activity is expressed as arbitrary densitometric units. Data represent the mean $\pm$ SD of three independent assays. As control, the amounts of IKKα were determined by immunoblotting with anti-IKKα Ab (Upper panel). (E) Receptors and intracellular pathways involved in the VIP and PACAP regulation of NF-κB nuclear translocation and IKK activity. Primary microglia were activated with LPS (500 ng/ml) in the absence (lane 1) or presence of VIP (10^{-8} M, lane 2) or forskolin (Forsk; 10^{-6} M, lane 5). VPAC1 antagonist (10^{-7} M, lanes 3) or H89 (100 ng/ml, lane 4) was added simultaneously with VIP (10^{-8} M). NF-κB binding for TNF-α and iNOS promoters was analyzed 1 h after stimulation by EMSA. After 1 h incubation, nuclear proteins were extracted, and Western blot analysis was performed for p50, p65, and phosphorylated IkB (P-IkB) in nuclear extracts. 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.

reduction in mRNA levels. Now the obligated question is how VIP and PACAP regulate such a wide spectrum of inflammatory mediators. The answer to this question could be found in the fact that gene activation of most of these factors is largely dependent on the activation of the pleiotropic transcription factor NF-κB [54]. NF-κB consists mostly of p50/p65 heterodimers that are complexed to the inhibitor IkB in the cytoplasm of 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 the p50/p65 heterodimers, which bind to regulatory sequences in a variety of target genes [54]. The present study indicates that VIP and PACAP inhibit LPS-induced p65 nuclear translocation and its subsequent binding to the κB motifs from promoters of TNF-α, IL-1β, IL-6, and iNOS in microglia. 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. Several reports have described a similar, inhibitory effect of VIP and PACAP on the NF-κB complex regulating other proinflammatory mediators in activated macrophages/monocytes [26, 27, 31, 40] and microglia [42]. Whereas VIP/PACAP inhibition of NF-κB binding in activated microglia is entirely cAMP-dependent, previous reports have demonstrated that in the case of activated macrophages and monocytes, the inhibition of NF-κB nuclear translocation by both neuropeptides is mediated through a cAMP-independent mechanism [26, 27, 31, 40]. These findings indicate that although microglia are the ontogenetic and functional equivalents of monocyte/macrophage lineage in the CNS, some differences exist between these cells in the transduction pathways used by VIP/PACAP in the regulation of the production of proinflammatory factors. In this sense, we have shown that similar to the effect on cytokine production, VIP and PACAP regulate LPS-induced production of proinflammatory chemokines [i.e., macrophage-inflammatory protein -1α (MIP-1α), MIP-2, regulated on activation, normal T expressed and secreted, monocyte

chemoattractant protein-1, and MIP-1 β] in microglia through a mechanism entirely cAMP-dependent [42].

Activation of microglia and subsequent production of proinflammatory and cytotoxic factors (i.e., reactive nitrogen and oxygen intermediates) have been attributed to increased neurotoxicity in in vitro neuron-microglia cultures treated with LPS or other inflammatory agents [55–57], suggesting that microglia-derived factors such as TNF- α , IL-1 β , IL-6, and NO are important mediators of inflammation-mediated neurodegeneration. Therefore, the VIP/PACAP-mediated inhibition on the production of proinflammatory mediators by activated microglia may have a therapeutic potential in pathological conditions of the CNS where an uncontrolled inflammatory response is involved, i.e., mechanical injury of brain [1–3]. However, the involvement of microglia-derived factors in neurodegeneration is not so simple. The reaction of the CNS to trauma is a predeterminant of the ability of the CNS to recover. Increase in the levels of proinflammatory cytokines is a normal and early feature of the CNS response to trauma [17–19]. However, it remains controversial as to whether inflammation in the injured CNS serves a beneficial or detrimental purpose [58, 59]. Several reports demonstrate that the administration of inflammatory cytokines to injured areas was neuroprotective or was the promoter to the regeneration of axons [58, 59]. It also has been reported that blood-derived macrophages modify the properties of the CNS white matter near mechanical lesions to convert a nonpermissive state to promoting axon growth [60]. In contrast, a number of studies have shown that the involvement of proinflammatory mediators such as TNF, IL-1 β , and NO in the mediation of neuronal and oligodendroglial death exists (reviewed in refs. [1–3, 58]), that the treatment with anti-inflammatory agents limits CNS damage and improves recovery after blunt spinal cord trauma [61], and that a correspondence of the number of macrophages/microglia with the amount of tissue damage exists [62]. It has been proposed that these differences in the role of inflammation in the CNS could be structure/region-specific and/or dependent of the varied stages of neuronal degeneration [63, 64]. In addition, inflammation in the CNS likely leads to the evolution of astrocyte reactivity and the multiple beneficial or detrimental effects of astrogliosis [58]. In this sense, we have recently demonstrated that both neuropeptides prevent in vitro and in vivo LPS- and mechanical trauma-induced neuronal cell death, probably by inhibiting the production of proinflammatory mediators by activated microglia (Delgado et al., submitted for publication). Therefore, at least in this study, the use of an anti-inflammatory factor, such as VIP or PACAP, avoids neurodegeneration. Probably, this neuroprotective effect of VIP/PACAP is brain region-dependent, as in comparison with other brain regions, the therapeutic effect of both peptides was much more efficient in the mesencephalic, especially around the periventricular nucleus, caudate putamen, and substantia nigra, where there exists a higher proportion of microglia. It is interesting that the VIP/PACAP-protective effect was especially selective for degeneration of dopaminergic neurons in the substantia nigra, where selective and progressive neuronal loss is characteristic of Parkinson's disease. In fact, recent experiments performed in our laboratory have demonstrated a neuroprotective role of VIP and PACAP on a murine model of Parkinson's disease by blocking

microglia activation (Delgado et al., submitted for publication). Therefore, we postulate that the administration of VIP or PACAP following acute trauma of the CNS could be beneficial, at least in certain regions of the brain, preventing neuronal cell loss in surrounding areas to the lesion. In addition, our study invites important future directions, including the possible therapeutic role of VIP in brain disorders, such as multiple sclerosis, Parkinson's and Alzheimer's diseases, and AIDS dementia, where inflammatory response is uncontrolled. At this point, interesting questions include: Which is the source of VIP and PACAP in CNS? Are VIP/PACAP produced under inflammatory conditions? Is intrinsic VIP able to prevent inflammatory events?

As far as we know, the unique VIP/PACAP source in the CNS is neuronal. Experiments performed in our laboratory have demonstrated that microglia are unable to produce VIP, even under inflammatory conditions (unpublished results). A systemic, acute inflammation, such as endotoxemia results in a dramatic increase in serum and peritoneal VIP levels [32], and neurons and lymphocytes are the main sources in such conditions. Exogenous administration of VIP increases fivefold the resistance to a determinant lethal dose of endotoxin [32]. In addition, a recent report has demonstrated the involvement of endogenously produced VIP in the survival of mice to endotoxic shock [65]. Although data are not available regarding the production of VIP on inflammatory events in the CNS, we can assume that during endotoxemia, certain brain regions, especially the circumventricular organs and parenchymal structures surrounding them, are seriously affected by endotoxin, occurring as a massive neurodegeneration, and similar to peripheral tissues, endogenous-produced VIP/PACAP could participate, together with other anti-inflammatory factors, in the neuroprotection of these areas. The effectiveness of such factors will depend on the balance of proinflammatory/anti-inflammatory mediators.

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