Vasoactive Intestinal Peptide and Pituitary Adenylate Cyclase-Activating Polypeptide Enhance IL-10 Production by Murine Macrophages: In Vitro and In Vivo Studies[1]

Mario Delgado,*† Ernesto J. Munoz-Elias,* Rosa P. Gomariz,† and Doina Ganea[2]*

Vasoactive intestinal peptide (VIP), a neuropeptide present in the lymphoid microenvironment, and the structurally related pituitary adenylate cyclase-activating polypeptide (PACAP) act as potent anti-inflammatory agents that inhibit the function of activated macrophages and TH cells. Previous reports showed that VIP/PACAP inhibit IL-6 and TNF-α production in LPS-stimulated macrophages. The present study reports on the effect of VIP/PACAP on IL-10 production. Although VIP/PACAP do not induce IL-10 by themselves, they enhance IL-10 production in LPS-stimulated macrophages. The specific VPAC1 receptor mediates the stimulatory effect of VIP/PACAP, and cAMP is the major second messenger involved. VIP/PACAP increase IL-10 mRNA in LPS-stimulated cells, and the effect of transcriptional and protein synthesis inhibitors indicates de novo IL-10 production. Electromobility shift assays show that VIP/PACAP induce an increase in nuclear cAMP response element (CRE)-binding complexes, with CRE binding protein as the major active component. Treatments with either a VPAC1 antagonist or a protein kinase A inhibitor abolish IL-10 stimulation and, concomitantly, the increase in CRE binding. Effects similar to the in vitro stimulation of IL-10 were obtained in vivo in mice treated with LPS and VIP or PACAP. The neuropeptides induce increased levels of IL-10 in both serum and peritoneal fluid, and increased expression of the IL-10 mRNA in peritoneal exudate cells. The stimulation of IL-10 production in activated macrophages represents a novel anti-inflammatory activity of VIP and PACAP, which presumably acts in vivo in conjunction with the inhibition of proinflammatory cytokines such as IL-6 and TNF-α to reduce the magnitude of the immune response. ***The Journal of Immunology*****, 1999, 162: 1707–1716.**

The generation of an immune response involves the activation of effector cells such as macrophages, neutrophils, and T lymphocytes and the subsequent production of cytokines, chemokines, and reactive oxygen and nitrogen intermediates. The activated macrophages are widely recognized as cells that play an important role in inflammatory processes as well as in the initiation, maintenance, and control of specific immune responses. In response to LPS and other activating agents, macrophages secrete nitric oxide and proinflammatory cytokines, such as TNF-α, IL-1β, IL-6, and IL-12, and immunomodulatory cytokines such as TGF-β1 and IL-10 (1). Since the intensity and duration of an inflammatory process depend on the local balance between pro- and anti-inflammatory factors, the so-called macrophage-deactivating factors have received considerable attention lately (2–6).

Vasoactive intestinal peptide (VIP)[3] and the pituitary adenylate cyclase-activating polypeptide (PACAP) are two multifunctional neuropeptides whose primary immunomodulatory function is anti-inflammatory in nature. VIP and PACAP have been shown to inhibit cytokine production and proliferation in T cells (reviewed in Ref. 7) and to inhibit macrophage phagocytosis, respiratory burst, and chemotaxis (reviewed in Ref. 8). In agreement with their anti-inflammatory role, VIP/PACAP were recently reported to inhibit IL-6 and TNF-α production in LPS-stimulated macrophages (9–11, 63), and to protect mice from endotoxic shock, presumably through the inhibition of endogenous TNF-α and IL-6 (12).

The immunological actions of VIP and PACAP are exerted through a family of VIP/PACAP receptors that have been recently reclassified (13): VPAC1 and VPAC2, which exhibit similar affinities for the two neuropeptides and activate primarily the adenylate cyclase system, and PAC1, which exhibits a 300- to 1000-fold higher affinity for PACAP than for VIP and activates both the adenylate cyclase and phospholipase C systems (reviewed in Ref. 14). Murine peritoneal macrophages were shown to possess VPAC1 and PAC1 (15–17).

IL-10, one of the major anti-inflammatory cytokines, was initially described as a Th2 product that inhibits the secretion of Th1-derived cytokines through the down-regulation of the Ag-presenting function of professional APCs (18, 19). In addition to T cells, activated monocytes/macrophages serve as a major IL-10 source, especially in response to LPS stimulation (20, 21). IL-10 inhibits several macrophage functions, such as oxidative burst, phagocytosis, nitric oxide production, and cytokine production (4, 20, 22–25).

Based on their effect on macrophage function, both the neuropeptides VIP/PACAP and the cytokine IL-10 could be classified as macrophage-deactivating factors. However, in addition to their

*Department of Biological Sciences, Rutgers University, Newark, NJ 07102; and †Departamento Biologia Celular, Universidad Complutense, Madrid, Spain

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[2] Address correspondence and reprint requests to Dr. Doina Ganea, Department of Biological Sciences, Rutgers University, 101 Warren Street, Newark, NJ 07102. E-mail address: dganea@andromeda.rutgers.edu

[3] Abbreviations used in this paper: VIP, vasoactive intestinal peptide; PACAP, pituitary adenylate cyclase-activating polypeptide; VPAC1, type 1 VIP receptor; VPAC2, type 2 VIP receptor; GRF, growth hormone-releasing factor; H89, *N*-[2-(*p*-bromocinnamyl-amino)ethyl]-5-iso-quinolinesulfonamide; EMSA, electrophoretic mobility shift assay; CRE, adenosine 3′,5′-monophosphate-responsive element; CREB, adenosine 3′,5′-monophosphate-responsive element-binding protein; PAC1, pituitary adenylate cyclase-activating polypeptide receptor; PKC, protein kinase C; PKA, protein kinase A.

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direct inhibitory effect on cytokine production by activated T cells and macrophages, VIP/PACAP may contribute to the down-regulation of the immune response through the induction of anti-inflammatory cytokines, particularly IL-10. In the present study we examine the in vitro and in vivo effects of VIP/PACAP on IL-10 protein and mRNA levels in LPS-stimulated peritoneal macrophages and investigate the molecular mechanisms involved.

Materials and Methods

Reagents

Synthetic VIP, PACAP38, VIP_{1-12}, and VIP_{10-28} were purchased from Novabiochem (Laufelfingen, Switzerland). 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 donated by Dr. Patrick Robberecht (Universite Libre de Bruxelles, Brussels, 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 gift from Drs. Ann Welton and David R. Bolin (Hoffmann-La Roche, Nutley, NJ). The synthetic PAC1 agonist maxadilan was a gift from Dr. Ethan A. Lerner (Massachusetts General Hospital, Charlestown, MA). The VPAC2/PAC1 antagonist $PACAP_{6-38}$, secretin, and glucagon were obtained from Peninsula Laboratories (Belmont, CA). Oligonucleotides were synthesized by the Oligonucleotide Synthesis Service of Rutgers University (Newark, NJ). Murine rIL-10 and capture and biotinylated anti-murine IL-10 mAbs were purchased from PharMingen (San Diego, CA). LPS (from *Escherichia coli* 055:B5), calphostin C, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS substrate), forskolin, PGE_2, protease inhibitors, PMSF, EDTA, glycine, protein G-agarose, glycerol, EGTA, DTT, and avidin-peroxidase were purchased from Sigma (St. Louis, MO), and *N*-[2-(*p*-bromocinnamyl-amino)ethyl]-5-iso-quinolinesulfonamide (H89) was obtained from ICN Pharmaceuticals (Costa Mesa, CA). Abs against CREB and c-Jun were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). All reagents were dissolved in endotoxin-free distilled water and diluted in serum-free RPMI 1640 (certified as endotoxin-free; Life Technologies, Gaithersburg, MD).

Preparation of macrophages and cell cultures

Mouse peritoneal macrophages were elicited by i.p. injection of 2 ml of 4% Brewer's thioglycolate medium (Difco, Detroit, MI) into male BALB/c mice (aged 6–10 wk; National Cancer Institute, Frederick, MD). Peritoneal exudate cells were obtained 72 h after injection by peritoneal lavage with 4 ml of ice-cold RPMI 1640 medium. Peritoneal exudate cells containing lymphocytes and macrophages were washed twice and resuspended in ice-cold RPMI 1640 medium supplemented with 2% heat-inactivated FCS (Atlanta Biologicals, Norcross, GA; certified as containing endotoxin levels $<$10 EU/ml) containing 10 mM HEPES buffer, 1 mM pyruvate, 0.1 M nonessential amino acids, 2 mM glutamine, 50 mM 2-ME, 100 U/ml penicillin, and 10 μg/ml streptomycin (RPMI 1640 complete medium). Cells were seeded in flat-bottom 96-well microtiter plates (Corning Glass, Corning, NY) at 8×10^4 cells/well in a final volume of 200 μl. The cells were incubated at 37°C for 2 h to allow adherence to plastic, and nonadherent cells were removed by repeated washing with RPMI 1640 medium. At least 96% of the adherent cells were macrophages as judged by morphologic and phagocytic criteria. Macrophage monolayers were incubated with RPMI complete medium and stimulated with LPS (0.5 μg/ml) in the presence or the absence of VIP or PACAP38 (from 10^{-12} to 10^{-6} M) at 37°C in a humidified incubator with 5% CO_2. Cell-free supernatants were harvested at the designated time points and kept frozen (−20°C) until determination of IL-10 production by ELISA.

In vivo quantitation of IL-10 production

Male BALB/c mice (6–10 wk old) received a single i.p. dose of LPS (100 μg/mouse) in the presence or the absence of different amounts of VIP or PACAP (from 0.5–10 nmol/mouse). After different time periods (2–8 h), blood was removed through cardiac puncture, and peritoneal exudate was obtained by peritoneal lavage with 4 ml of ice-cold RPMI 1640 medium. The blood samples were allowed to clot for 1 h at room temperature; serum was obtained and kept frozen until used in IL-10 ELISA. The peritoneal suspension was centrifuged 5 min at $1800 \times g$, and cell-free supernatants (peritoneal fluid) were harvested and assayed for IL-10 production. The peritoneal cells were subjected to Northern blot analyses as described below.

Cytokine determination: ELISA assay for IL-10

The amounts of IL-10 present in culture supernatants, peritoneal fluid, and serum were determined using the murine IL-10 sandwich ELISA essentially as described previously (26), with the IL-10-specific mAb JES3–9D7 as capture Ab and the biotinylated anti-IL-10 mAb JES-12G8 as detection Ab. The sensitivity of the IL-10 ELISA was 10 pg/ml. The assay was specific for IL-10, with other recombinant cytokines (IL-1, IL-2, IL-4, IL-7, IL-6, IL-12, and IFN-γ) not binding above background levels.

RNA extraction and Northern blot analysis

Northern blot analysis was performed according to standard methods. Macrophage monolayers (10^7 cells/well in six-well tissue culture plates) were cultured in 5 ml of medium and stimulated with LPS (0.5 μg/ml) in the absence or the presence of VIP and PACAP (10^{-8} M) for different time periods at 37°C. Total RNA was extracted by the acid guanidinium-phenol-chloroform method, electrophoresed on 1.2% agarose-formaldehyde gels, transferred to Nytran membranes (Schleicher and Schuell, Keene, NJ), and cross-linked to the nylon membrane using UV light. The probes for murine IL-10 and β-actin were generated by RT-PCR as described previously (26). Oligonucleotides were end labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. The RNA-containing membranes were prehybridized for 16 h at 42°C and then hybridized at 60°C for 16 h with the appropriate probes. The membranes were washed twice in 2× SSC containing 0.1% SDS at room temperature (20 min each time), once at 37°C for 20 min, and once in 0.1× SSC containing 0.1% SDS at 50°C (20 min). The prehybridization and hybridization buffers were purchased from 5 Prime-3 Prime (Boulder, CO). The membranes were exposed to x-ray films (Eastman Kodak, Rochester, NY). Signal quantitation was performed in a PhosphorImager SI (Molecular Dynamics, Sunnyvale, CA).

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared by a mini-extraction procedure. Briefly, peritoneal macrophages were plated at a density of 10^7 cells in six-well plates, stimulated, washed twice with ice-cold PBS/0.1% BSA, and scraped off the dishes. The cell pellets were homogenized with 0.4 ml of buffer A (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 1 mM NaN_3). After 15 min on ice, Nonidet P-40 was added to a 0.5% final concentration, the tubes were gently vortexed for 15 s, and nuclei were sedimented and separated from cytosol by centrifugation at $12{,}000 \times g$ for 40 s. The pelleted nuclei were washed once with 0.2 ml of ice-cold buffer A, and the soluble nuclear proteins were released with 0.1 ml of 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 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 1 mM NaN_3). After incubation for 30 min on ice, the nuclear proteins were separated from the remaining nuclei by centrifugation for 10 min at 14,000 rpm at 4°C. The protein concentration in the supernatants was determined by the Bradford method, and aliquots were stored at −80°C for later use in EMSAs.

Oligonucleotides corresponding to the CRE (5′-TTTATCCACGTCATTATGACC-3′; nucleotides 1201–1221), and NF-κB (5′-CTCTCGGGGTTTCCTTTGGG-3′; nucleotides 1069–1088) motifs of the IL-10 promoter were synthesized (27). Oligonucleotides were annealed by incubation for 5 min at 85°C in 10 mM Tris-HCl (pH 8.0), 5 mM NaCl, 10 mM $MgCl_2$, and 1 mM DTT. Fifty nanograms of the double-stranded oligonucleotides were end labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. For EMSAs with macrophage nuclear extracts, 20,000–50,000 cpm of the double-stranded oligonucleotide, corresponding to approximately 0.5 ng, was used for each reaction. Binding reaction mixtures (15 μl) were set up containing 0.5–1 ng of DNA probe, 5 μg of nuclear extract, 2 μg of 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 reaction was incubated on ice for 15 min before adding the probe, followed by 20 min at room temperature. Samples were loaded onto 4% nondenaturing polyacrylamide gel 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 (Clifton, NJ), drying under vacuum at 80°C, and autoradiography. In competition and Ab supershift experiments, the nuclear extracts were incubated for 15 min at room temperature with Ab (1 μg) or competing oligonucleotide (50-fold excess) before addition of the labeled probe.

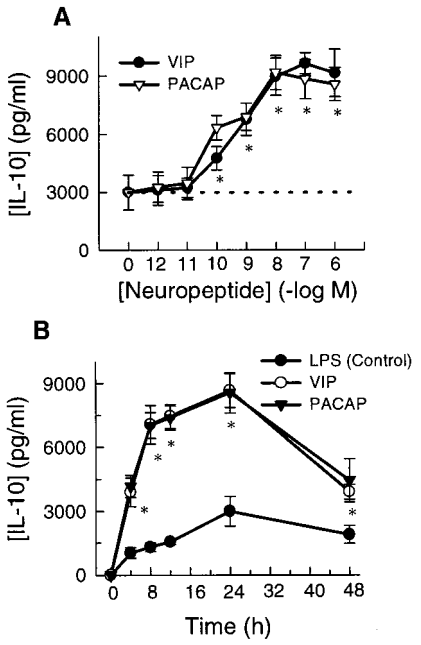

FIGURE 1. VIP and PACAP stimulate IL-10 production by LPS-treated macrophages. *A*, Dose-response curve for the stimulatory effect of VIP and PACAP on IL-10 production. Peritoneal macrophages were stimulated with LPS (0.5 μg/ml) and a concentration range of either VIP or PACAP for 24 h. The IL-10 contents of culture supernatants were determined by ELISA. The dotted line represents the IL-10 value in LPS-stimulated macrophages cultured in the absence of VIP/PACAP. *B*, Time course for the stimulation of IL-10 production by VIP and PACAP. Macrophages were stimulated with LPS (0.5 μg/ml) in the absence or the presence of 10^{-8} M VIP or PACAP, and supernatants harvested at different times were assayed for IL-10 by ELISA. Control cultures were incubated with LPS alone. For *A* and *B*, cells cultured in the absence of LPS with or without VIP/PACAP did not produce detectable levels of IL-10 (<10 pg/ml). Each result is the mean ± SD of four separate experiments. Each sample was assayed in duplicate. *, $p < 0.001$ with respect to control cultures with LPS alone.

Statistical analysis

All values are expressed as the mean ± SD of the number of experiments. In each experiment samples were assayed in duplicate. Comparisons between groups were made using Student's *t* test followed by Scheffe's *F* test, with $p < 0.05$ as the minimum significant level.

Results

VIP and PACAP stimulate LPS-induced IL-10 production

Peritoneal macrophages cultured in the presence of VIP or PACAP (from 10^{-7} to 10^{-11} M) did not secrete detectable levels of IL-10. However, the addition of VIP/PACAP to LPS-stimulated cultures resulted in a significant increase in IL-10 production. VIP and PACAP dose-dependently increased the amount of IL-10 released in response to LPS, showing maximal effects at 10^{-6}–10^{-8} M (Fig. 1*A*). The time curves indicate that VIP and PACAP stimulate IL-10 release as early as 4 h, with a maximum stimulatory effect at 24 h (Fig. 1*B*).

The experiments reported above were performed with peritoneal adherent cells. Since peritoneal lymphocytes, especially $CD5^+$ B cells, which secrete IL-10 (28), may contaminate the adherent cell population, we determined the effect of VIP/PACAP on the IL-10 production by LPS-stimulated nonadherent peritoneal cells. Nonadherent peritoneal cells obtained after the 2-h incubation in tissue culture plates were stimulated with LPS in the presence or the absence of VIP and PACAP. Supernatants collected 24 h later showed no detectable IL-10 (data from three experiments, not shown). In addition, we found no differences in the amount of IL-10 produced by thioglycolate-induced, freshly isolated peritoneal cell suspensions (~60–80% macrophages, 20–40% lymphocytes) and monolayer cell preparations containing about 96% macrophages, confirming that the peritoneal lymphocytes do not contribute to the IL-10 production in response to LPS.

The stimulation of IL-10 production by VIP and PACAP is mediated through VPAC1

Next we investigated whether the stimulatory effect of VIP/PACAP could be related to the occupancy of specific receptors. First, we compared the effect of VIP/PACAP to that of secretin, glucagon, and the VIP and PACAP fragments VIP_{1-12}, VIP_{10-28}, and $PACAP_{6-38}$. IL-10 production was not affected by different concentrations of secretin and glucagon (10^{-7}–10^{-9} M; Fig. 2*A*). The two VIP fragments and $PACAP_{6-38}$ failed to stimulate IL-10 production, suggesting that intact VIP and PACAP molecules are required for their stimulatory activity (Fig. 2*A*).

The nature of the VIP/PACAP receptors involved was investigated using specific receptor agonists and antagonists. We determined the effect of a newly described VPAC1 agonist (29), a VPAC2 agonist (Ro 25–1553) (30), and maxadilan, a specific PAC1 agonist (31), on LPS-induced IL-10 production. The VPAC1 agonist, but not the VPAC2 and PAC1 agonists, stimulated IL-10 release with a potency similar to that of VIP/PACAP (300% stimulation; Fig. 2*B*). In addition, we investigated the ability of $PACAP_{6-38}$, an antagonist specific for PAC1 and to a lesser degree for VPAC2 (32), and of a specific VPAC1 antagonist (33) to reverse the effects of VIP and PACAP. The VPAC1 antagonist reversed the effect of VIP/PACAP in a dose-dependent manner (Fig. 2*C*). In contrast, $PACAP_{6-38}$ did not reverse the stimulatory effect of VIP and PACAP (Fig. 2*D*). Neither the VPAC1 antagonist nor $PACAP_{6-38}$ significantly affected IL-10 levels in LPS-stimulated cells (Fig. 2, *C* and *D*). The simultaneous addition of VIP/PACAP and the VPAC1 agonist did not result in an additive effect (Fig. 2*E*). Together these results confirm the specificity of the VIP and PACAP stimulatory effect and suggest that both neuropeptides exert their action through VPAC1.

Time course for the stimulatory effect of VIP and PACAP on IL-10 production

In the experiments described to date VIP and PACAP were added at the same time as LPS. To determine the time interval required for the stimulatory effect of VIP/PACAP, we stimulated peritoneal macrophages with LPS and added 10^{-8} M VIP or PACAP at later times (from 0–18 h). Supernatants were collected 24 h after the addition of VIP/PACAP and were assayed for IL-10 production. The IL-10 levels were compared with controls containing LPS and no VIP/PACAP, which were harvested at similar time points as the VIP/PACAP-containing cultures. The addition of VIP and PACAP up to 12 h after LPS stimulation resulted in significant levels of stimulation (280–355%; Fig. 3*A*). Later additions (18 h) resulted in a lesser degree of stimulation (Fig. 3*A*). To determine the minimum time required for the stimulatory effect of VIP and PACAP, peritoneal macrophages were cultured with LPS in the presence or the absence of 10^{-8} M VIP or PACAP. The neuropeptides were removed by extensive washing at different times (from 5–180 min), and the cells were cultured in medium containing LPS without neuropeptides. Five-minute incubations resulted in significant stimulatory effects, and 15-min incubations led to maximum stimulation (Fig. 3*B*).

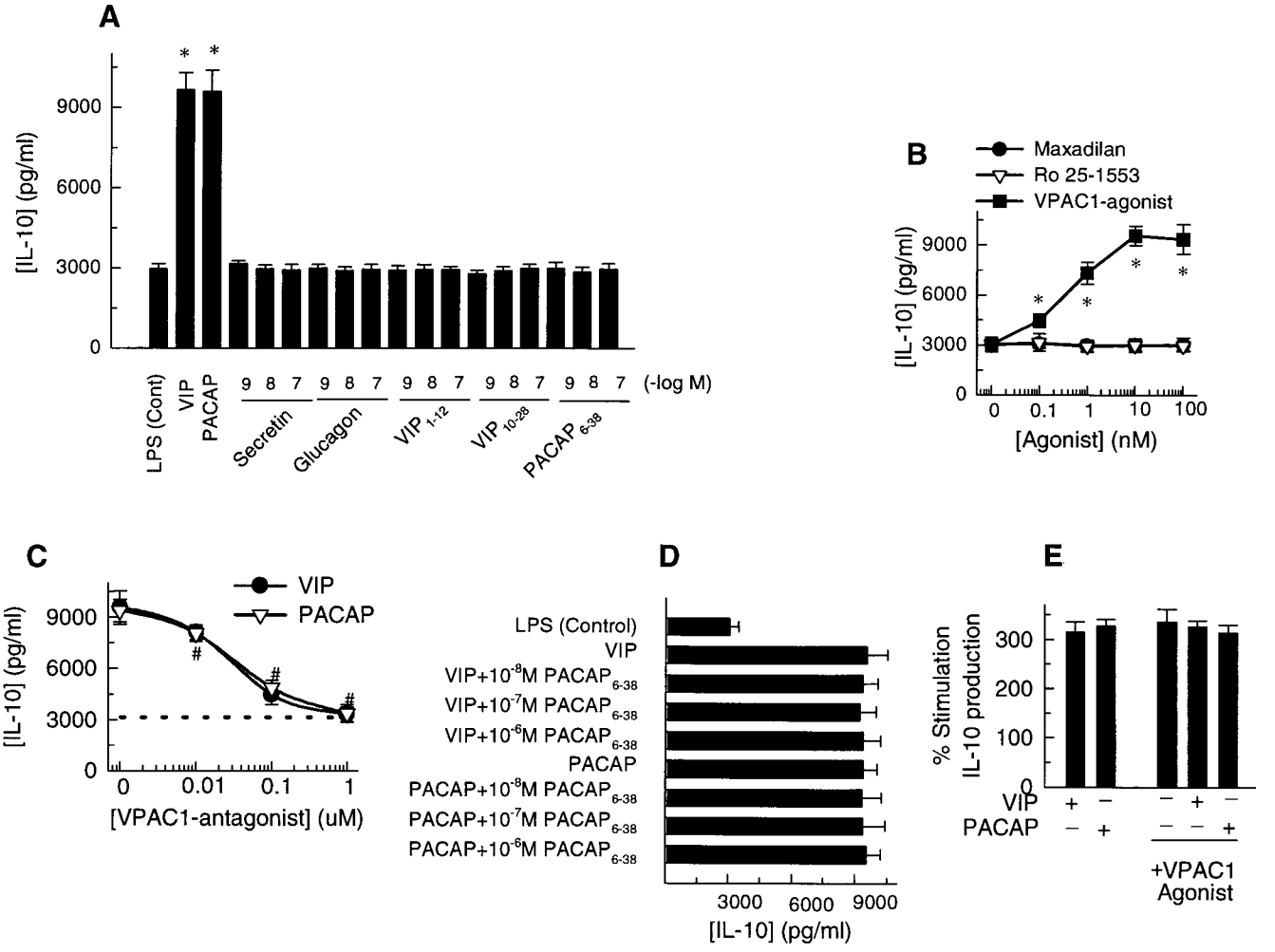

FIGURE 2. The effect of VIP/PACAP is mediated through VPAC1. *A* and *B*, Comparative effects of VIP, PACAP38, VIP-related peptides, VIP and PACAP fragments, and VIP and PACAP agonists on IL-10 production. Peritoneal macrophages were stimulated with LPS (0.5 μg/ml) in the presence or the absence of different concentrations of secretin, glucagon, VIP_{1-12}, VIP_{10-28}, and $PACAP_{6-38}$ (*A*) or 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; *B*). Supernatants were collected 24 h later and assayed for IL-10 by ELISA. Each result is the mean ± SD of five experiments. Each sample was assayed in duplicate. *, $p < 0.001$ with respect to control cultures with LPS alone. *C* and *D*, Effects of PAC1 and VPAC antagonists on the stimulatory effect of VIP and PACAP. Peritoneal macrophages were stimulated with LPS (0.5 μg/ml) 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} (*C*) or the PAC1/VPAC2 antagonist ($PACAP_{6-38}$; *D*). Supernatants were collected 24 h later and assayed for IL-10 by ELISA. The VPAC1 antagonist (10^{-6} M) and $PACAP_{6-38}$ (10^{-6} M) did not affect IL-10 production in LPS-treated macrophages (3166 ± 243 pg/ml for VPAC1 and 3055 ± 115 pg/ml for $PACAP_{6-38}$). The dotted line in *C* represents control values from cultures incubated with LPS alone (3033 ± 127 pg/ml). Each result is the mean ± SD of four experiments. Each sample was assayed in duplicate. *, $p < 0.001$ compared with samples treated with neuropeptides and without antagonists. *E*, Effect of the VPAC1 agonist on the stimulatory activity of VIP/PACAP. Macrophages were stimulated with LPS (0.5 μg/ml) and were treated with the VPAC1 agonist (100 nM) in the presence or the absence of VIP or PACAP (10^{-8} M). Supernatants were collected 24 h later and assayed for IL-10. The percent stimulation was calculated by comparison with controls containing LPS alone. Results are the mean ± SD of four experiments.

Intracellular pathways involved in the stimulation of IL-10 production by VIP and PACAP

To study the second messengers involved in the effect of VIP and PACAP, we investigated the effects of calphostin C (a PKC inhibitor) (34), H89 (a PKA inhibitor) (35), and forskolin and PGE_2 (two strict cAMP-inducing agents) (36) on the release of IL-10. Forskolin and PGE_2 stimulated IL-10 production similar to VIP and PACAP (Fig. 4*A*), suggesting the involvement of cAMP. The role of cAMP as second messenger is also supported by the effect of the PKA inhibitor H89. In the concentration range in which calphostin C (the PKC inhibitor) does not affect IL-10 production in LPS-stimulated cells (Fig. 4*B*, *right panel*), it does not reverse the stimulatory effect of VIP/PACAP (Fig. 4*B*, *left panel*). In contrast, H89 (the PKA inhibitor) reversed in a dose-dependent manner the stimulatory effect of VIP/PACAP (Fig. 4*B*, *center panel*). These results suggest that the stimulatory effect of VIP/PACAP on IL-10 production is mediated through increases in intracellular cAMP.

VIP and PACAP increase IL-10 production at the mRNA level

Having demonstrated that VIP and PACAP had a stimulatory effect on IL-10 production, we sought to determine whether this action occurred at a transcriptional level. We stimulated peritoneal macrophages with LPS in the presence or the absence of 10^{-8} M VIP or PACAP for 2, 4, 8, and 18 h, and total RNA was prepared and subjected to Northern blot analysis. Although no IL-10 mRNA was detectable in unstimulated cells (Fig. 5*A*), time-dependent increasing levels of IL-10 mRNA are present in LPS-stimulated cells

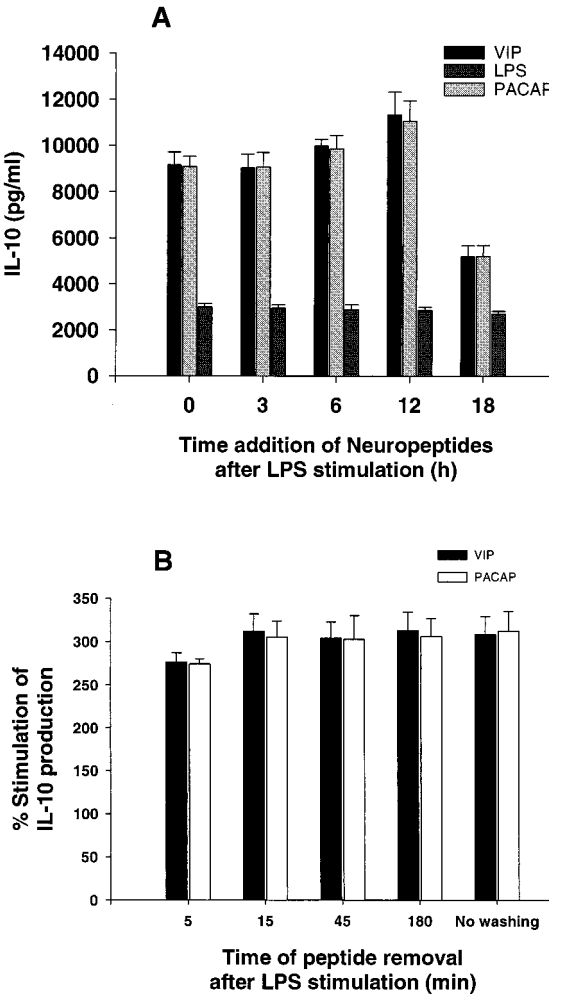

FIGURE 3. Time course for the stimulatory activity of VIP and PACAP. *A*, Macrophages were stimulated with LPS (0.5 μg/ml) at time zero, and VIP or PACAP (10^{-8} M) was added at different times after the initiation of the cultures. Supernatants were collected 24 h after the addition of the VIP/PACAP and assayed for IL-10. *B*, Peritoneal macrophages were stimulated with LPS (0.5 μg/ml) in the presence or the absence of VIP or PACAP (10^{-8} M). The neuropeptides were removed at different times by washing (three times with serum-free medium), and the cells were resuspended in complete medium containing LPS (0.5 μg/m) in the absence of neuropeptides and cultured for an additional 24 h. Control cultures containing only LPS were washed and recultured under the same conditions. Supernatants were collected, and the IL-10 content was determined by ELISA. The percent stimulation was calculated by comparing each experimental culture with its control. Each result is the mean ± SD of four experiments.

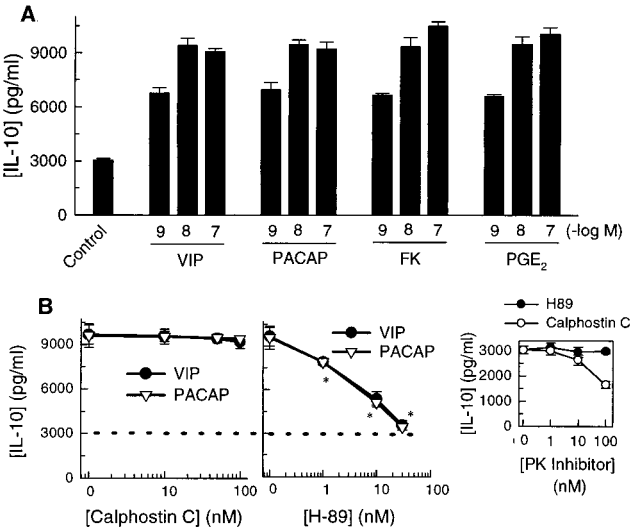

FIGURE 4. cAMP as a second messenger for the stimulatory activity of VIP and PACAP on IL-10 production. *A*, Effects of various cAMP-inducing agents. Peritoneal macrophages were stimulated with LPS (0.5 μg/ml) in the presence or the absence of different concentrations of VIP, PACAP, forskolin (FK), or PGE_2. Twenty-four-hour culture supernatants were assayed for IL-10 by ELISA. Each result is the mean ± SD of five experiments. *B*, Comparative effects of calphostin C (a PKC inhibitor) and H89 (a PKA inhibitor) on the stimulatory activity of VIP and PACAP. Macrophages were stimulated with LPS (0.5 μg/ml), LPS plus VIP (10^{-8} M), or LPS plus PACAP (10^{-8} M) in the absence or the presence of different concentrations of calphostin C or H89. Twenty-four-hour culture supernatants were assayed for IL-10 by ELISA. The dotted line represents control values from cultures incubated with LPS alone (2978 ± 139 pg IL-10/ml). Each result is the mean ± SD of five experiments. *, $p < 0.001$ with respect to neuropeptide-treated samples without protein kinase inhibitors.

(Fig. 5, *B* and *C*). At all time points, VIP and PACAP significantly increased the levels of specific IL-10 mRNA (Fig. 5, *B* and *C*). These results indicate that both neuropeptides exert their actions at the level of mRNA.

In addition, to confirm that VIP and PACAP stimulate the de novo synthesis of IL-10, LPS-stimulated macrophages were incubated with cycloheximide, a protein synthesis inhibitor, or with actinomycin D, a transcriptional inhibitor, in the presence or the absence of VIP or PACAP. Both cycloheximide and actinomycin D reduced IL-10 to undetectable levels (Fig. 6), suggesting that VIP and PACAP induce de novo transcription and synthesis of IL-10.

VIP and PACAP stimulate CREB binding

Although the IL-10 promoter contains a complex array of transactivating binding sites, the cAMP-responsive element appears to be essential for maximal IL-10 transcription (27, 37). In addition, it has been described that cAMP-elevating drugs, such as PGE_2 and dibutyryl cAMP, up-regulate IL-10 production by macrophages (37–40). To investigate whether VIP/PACAP affect CRE binding, we used EMSAs. Stimulation of macrophages with LPS led to a time-dependent increase in CRE binding compared with that in unstimulated cells, and treatment with VIP and PACAP significantly increased this binding (Fig. 7*A*). The binding specificity was confirmed using homologous (CRE) and nonhomologous (NF-κB) oligonucleotides as competitors (Fig. 7*A*). Ab supershift experiments were performed to determine the composition of the CRE-binding factors. In VIP/PACAP-treated cells, the majority of the complex was supershifted by an anti-CREB Ab, whereas no supershift was observed using an anti-c-Jun Ab (Fig. 7*B*), indicating the presence of CREB in the CRE-binding complexes.

Since the stimulatory effect of VIP and PACAP on IL-10 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 CRE binding. The stimulatory activity of VIP on CRE binding was completely reversed by the VPAC1 antagonist and H89 (Fig. 7*C*). These results suggest that the stimulation of CRE binding by VIP is mediated through VPAC1 and the subsequent elevation of cAMP levels. This is supported by the fact that forskolin affected CRE binding the same way as VIP (Fig. 7*C*).

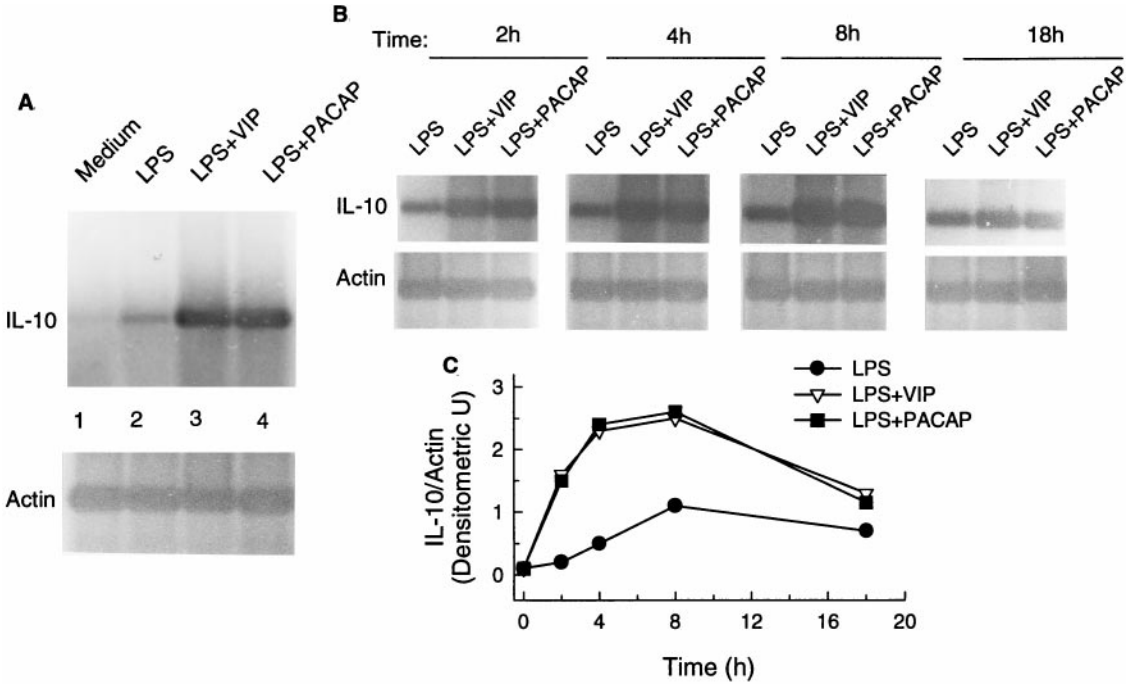

FIGURE 5. VIP and PACAP stimulate IL-10 transcription. *A* and *B*, Macrophages were stimulated with LPS (0.5 μg/ml) and incubated with or without VIP or PACAP (10^{-8} M) for 8 h (*A*) and for 2, 4, 8, and 18 h (*B*). The expression of IL-10 and β-actin mRNA was analyzed by Northern blot analysis at the indicated time points. *C*, Results are expressed in arbitrary densitometric units normalized for the expression of β-actin in each sample. One representative experiment of three is shown.

VIP and PACAP augment IL-10 production in vivo

An attempt was made to reproduce the in vitro observations in vivo. The i.p. injection of LPS (100 μg) resulted in elevation of IL-10 in serum and peritoneal exudate fluid (Fig. 8*A*). Treatment of mice with VIP or PACAP significantly enhanced the LPS-induced IL-10 level in serum and peritoneal fluid (Fig. 8*A*). The in vivo effects of VIP and PACAP were dose dependent, with a maximum effect at 5–10 nmol/animal (Fig. 8*B*). In addition, VIP and PACAP significantly stimulated IL-10 transcription in freshly isolated peritoneal exudate cells, 2 and 4 h after the LPS challenge (Fig. 8*C*).

Discussion

VIP and PACAP are potent anti-inflammatory agents that downregulate the activation of T cells and macrophages (7, 8, 41). Several other properties, especially vasodilation, make them of potential benefit in metabolically unfavorable circumstances such as hypoxia or ischemia (42). Recently, VIP and PACAP were shown to modulate the macrophage secretion of proinflammatory cytokines such as TNF-α and IL-6 (9, 11, 63). This might have clinical relevance, since these cytokines are involved in the detrimental effects of ischemia-reperfusion and septic shock (1, 43, 44). The present study shows a novel property of VIP/PACAP that might contribute to their anti-inflammatory effects, e.g., the stimulation of IL-10 production in LPS-activated macrophages. The stimulatory effect is dose dependent within a wide range of neuropeptide concentrations (10^{-6}–10^{-10} M), with the maximum effect being observed at 10^{-8} M. This is the dose range in which VIP and PACAP modulate several other immunological functions (7–9, 41).

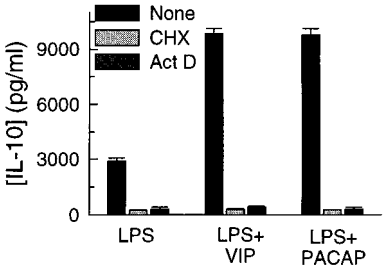

FIGURE 6. Effects of cycloheximide and actinomycin D on the stimulatory activity of VIP and PACAP. Macrophages were stimulated with LPS (0.5 μg/ml), LPS plus VIP (10^{-8} M), or LPS plus PACAP (10^{-8} M) for 3.5 h before the addition of actinomycin D (ActD; 5 μg/ml) or for 8 h before the addition of cycloheximide (CHX; 1 μg/ml). Supernatants were collected 8 h later and assayed for IL-10 production by ELISA. Each result is the mean ± SD of four experiments.

The time course indicates that, similar to the effect on IL-6 and TNF-α (25, 63), VIP and PACAP affect IL-10 production rapidly, with 5- to 15-min incubations sufficient to achieve maximum stimulatory effects. In addition, the stimulation has a refractory period of 12 h, after which the stimulatory activity of VIP/PACAP is reduced significantly.

Of relevance is the fact that in the absence of LPS, VIP and PACAP do not stimulate IL-10 release. This observation indicates that VIP/PACAP can only amplify an endotoxin-generated signal and suggests that the in vivo action of VIP/PACAP may be restricted to cells that are actively involved in responding to Ags.

Similar to the effect on other cytokines such as IL-2, IL-6, TNF-α, and IL-12 (7, 27, 63)[4] the stimulation of IL-10 requires intact VIP/PACAP molecules. This is in agreement with previous reports showing that either C- or N-terminal truncations of VIP

[4] Delgado, M., E. J. Munoz-Elias, R. P. Gomariz, and D. Ganea. VIP and PACAP inhibit IL-12 production in LPS-stimulated macrophages: subsequent effect on T cell IFN-γ synthesis. *Submitted for publication.*

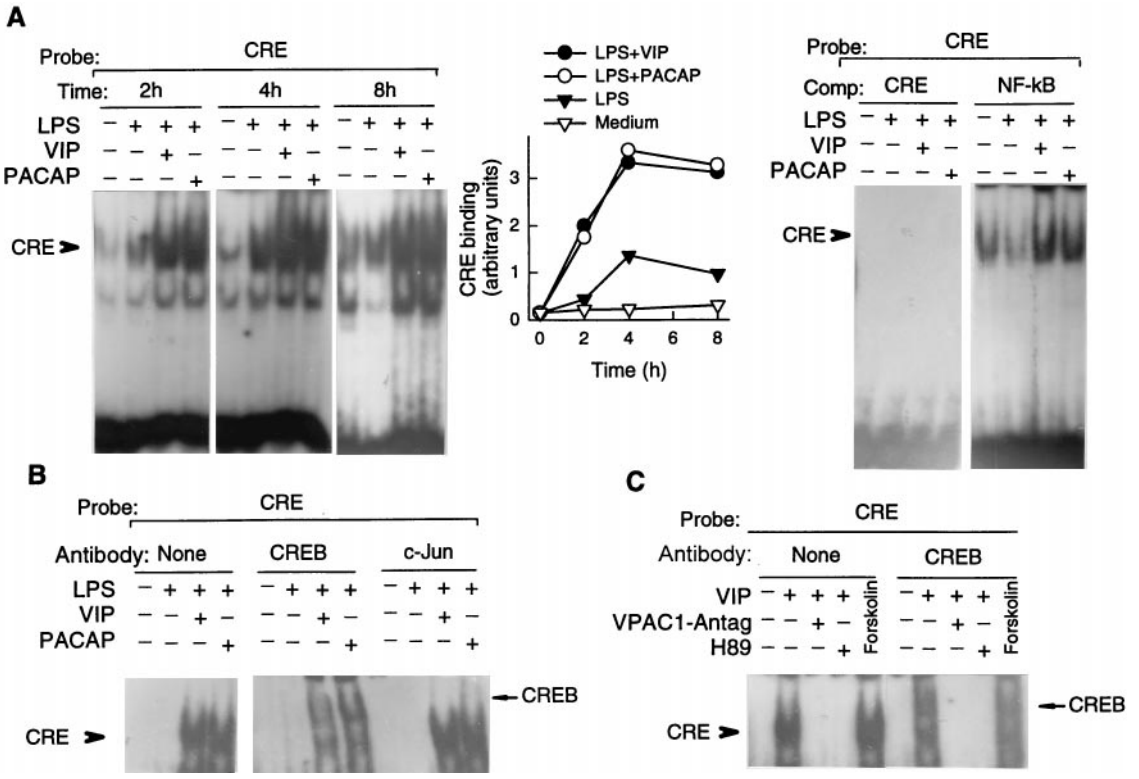

FIGURE 7. VIP and PACAP stimulate CREB binding through the VPAC1/cAMP pathway. *A*, Nuclear extracts were prepared from peritoneal macrophages incubated for 2, 4, and 8 h with LPS (0.5 μg/ml) in the presence or the absence of VIP or PACAP (10^{-8} M). CRE binding was assessed by EMSA using a radiolabeled oligonucleotide containing the murine CRE site of the IL-10 promoter. Specificity was determined by the addition of a 50-fold excess of unlabeled nonhomologous (NF-κB) or homologous (CRE) oligonucleotides to the nuclear extracts (Comp). *B*, Identification of the proteins bound to the CRE site using supershift analysis. Nuclear extracts (8-h incubation) were incubated with polyclonal Abs against CREB or c-Jun for 20 min before addition of the CRE probe. *C*, Nuclear extracts were prepared from macrophages incubated for 8 h with LPS (0.5 μg/ml; *lane 1*); LPS plus VIP (10^{-8} M; *lane 2*); LPS, VIP, and VPAC1 antagonist (10^{-6} M; *lane 3*); LPS, VIP, and H89 (100 nM; *lane 4*), or LPS plus forskolin (10^{-6} M; *lane 5*). The proteins bound to the CRE site were identified by supershift analysis. Similar results were observed in three independent experiments.

lead to significant losses in biological activity (45, 46). Peritoneal macrophages have been previously shown to express VPAC1 and PAC1 mRNA, and both high and low affinity VIP/PACAP binding sites (16, 17). In addition, we have recently reported that LPS-stimulated Raw 264.7 macrophages express mRNA for all three VIP/PACAP receptors (47). Our agonist studies suggest that VPAC1 mediates the stimulatory effect on IL-10. This is in agreement with Dewit et al. (11), who reported that a VPAC2 agonist has no effect on IL-10 production in human blood monocytes. The role of VPAC1 as the unique mediator in the effect on IL-10 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 (32), reverses the stimulatory effect of VIP/PACAP. Also, the VPAC1 antagonist blocked the effect of VIP/PACAP on CREB binding to the CRE site specific for the IL-10 promoter, supporting the involvement of VPAC1 in the stimulatory effect of VIP/PACAP on IL-10 gene expression.

The VPAC1 is coupled primarily to the adenylate cyclase system (14). To fully understand the mechanism of action of VIP and PACAP, it is important to clarify which transduction pathways are involved in the stimulation of IL-10 in macrophages. It has been reported that in monocytes/macrophages IL-10 production is stimulated by cAMP-inducing agents (37–40). In the present study forskolin and PGE_2, two strict cAMP-inducing agents (36), stimulate IL-10 release similar to VIP and PACAP. In addition, H89, a potent and selective PKA inhibitor (35), completely reverses the effect of VIP/PACAP. In contrast, calphostin C (a specific PKC inhibitor) (34) does not alter the stimulatory action of either VIP or PACAP. These results suggest that VIP/PACAP stimulate IL-10 production in macrophages through the cAMP/PKA pathway initiated through binding to VPAC1.

Previous experiments regarding VIP modulation of cytokine expression indicated different molecular mechanisms, i.e., transcriptional regulation for IL-2, IL-6, and TNF-α vs post-transcriptional regulation for IL-4 (9, 47, 48, 63). The present study indicates that the stimulatory effect of VIP and PACAP on IL-10 production occurs at the mRNA level. This is supported by the fact that VIP/PACAP induce increases in steady state IL-10 mRNA, and that the stimulatory effect on the IL-10 release is inhibited by both transcriptional and protein synthesis inhibitors. It remains to be established whether the augmentation of steady state IL-10 mRNA levels results from an increase in either or both the novo transcriptional rate and message stabilization.

Although the IL-10 promoter contains a complex array of transactivating binding sites, the CRE appears essential for maximal IL-10 transcription in monocytes/macrophages (27, 37). Since VIP and forskolin induce similar increases in CRE-binding nuclear proteins, and H89 reverses the effect of VIP on CRE binding, we propose that VIP/PACAP stimulate IL-10 gene expression in peritoneal macrophages through a cAMP-dependent increase in the functionally active transcriptional factor CREB. In this respect, VIP and PACAP were reported recently to increase CREB phosphorylation and CREB-regulated transcription in several cell types (49–51). Since VIP/PACAP do not promote IL-10 production in the absence of LPS, the sole increase in CRE-binding activity is probably insufficient to induce the expression of the IL-10 gene.

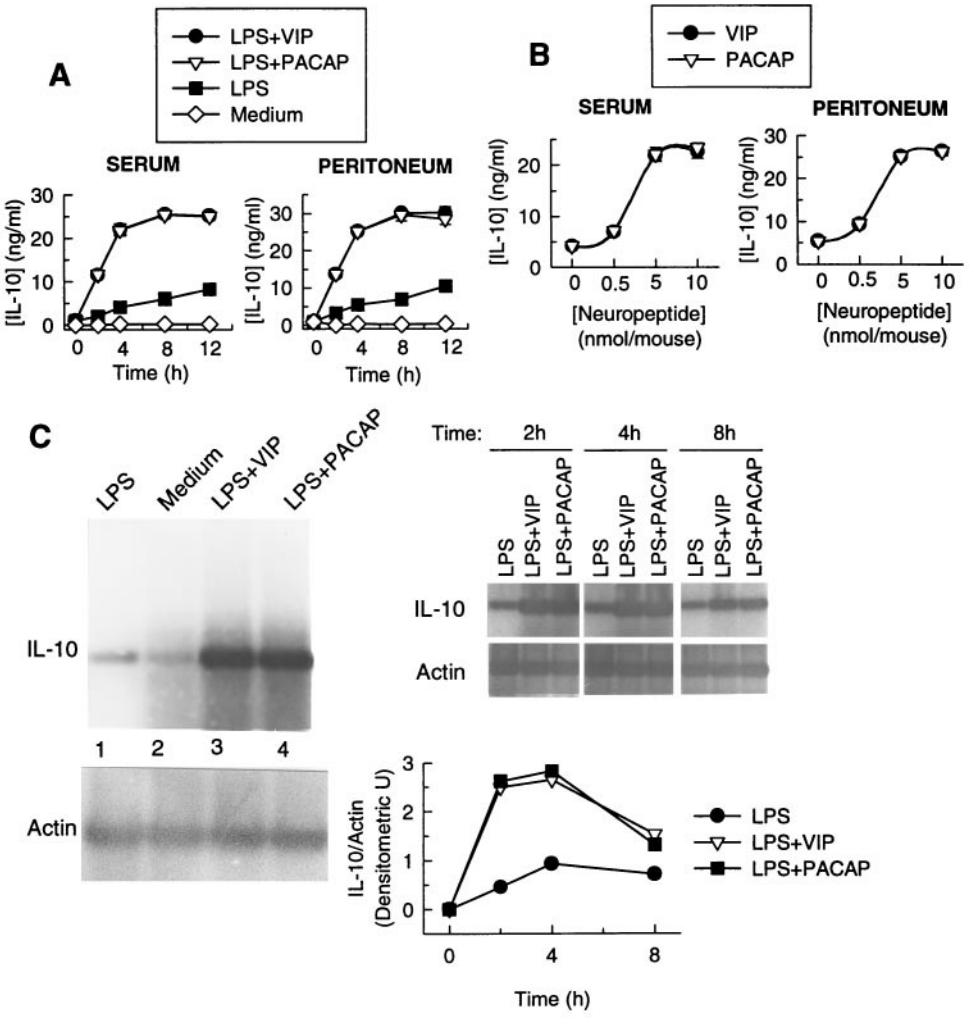

FIGURE 8. In vivo effects of VIP and PACAP on LPS-induced IL-10 production. *A*, Mice (groups of three) were injected i.p. with LPS (100 μg/mouse), LPS plus VIP (5 nmol/mouse), or LPS plus PACAP (5 nmol/mouse). Serum and peritoneal fluid were obtained at the indicated time points as described in *Materials and Methods* and were assayed for IL-10 by ELISA. Each result is the mean ± SD of three experiments. *B*, Mice (groups of three) were injected i.p. with LPS (100 μg/mouse) in the presence or the absence of different concentrations of VIP or PACAP (0.5–10 nmol/mouse). Serum and peritoneal fluid were obtained after 8 h and assayed for IL-10 by ELISA. Each result is the mean ± SD of three experiments. *C*, Expression of IL-10 mRNA in peritoneal exudate cells. Mice (groups of three) were injected i.p. with medium alone (control for basal IL-10 mRNA), LPS (100 μg/mouse), LPS plus VIP (5 nmol/mouse), or LPS plus PACAP (5 nmol/mouse), and peritoneal exudate cells were harvested at 2 h (*left panel*) and at 2, 4, and 8 h (*right panel*). The expression of IL-10 mRNA was determined by Northern blot analysis. Results (*lower panel*) are expressed in arbitrary densitometric units normalized for the expression of β-actin in each sample. One representative experiment of three is shown.

However, CREB could act in coordination with other transcriptional factors regulated by LPS, to induce maximal transcriptional activity for the IL-10 gene.

The necessity of several transcriptional factors acting in concert for the expression of the IL-10 gene may also explain the differences in the effects of VIP on IL-10 production in activated T cells vs activated macrophages. Previously we reported that VIP inhibits IL-10 expression in anti-CD3-stimulated T cells (26). This is in contrast to the present study, which indicates that VIP/PACAP stimulate IL-10 production in LPS-activated macrophages. In both cases, forskolin mimicked the effect of VIP, suggesting that the major transduction pathway was cAMP dependent. However, the nature of second messengers downstream from cAMP might be different in T cells and macrophages. Also, whereas in macrophages CREB induced by VIP/PACAP might act in concert with transcriptional factors induced by LPS, in T cells cAMP-dependent events such as the inhibition of JNK (52) might lead to the reduction in functionally active c-Jun protein and subsequent reduction in the transcriptional activity at the AP-1 site. Such events have been identified for the IL-2 promoter in anti-CD3-stimulated T cells treated with VIP (our unpublished observations).

The in vitro stimulatory effect of VIP/PACAP on IL-10 production correlates with the in vivo stimulation of both IL-10 expression and release in endotoxemic mice. VIP and PACAP both accelerate and increase the levels of circulating IL-10 and the IL-10 mRNA expression in peritoneal cells in endotoxemic mice. In contrast to the effect on IL-10, VIP and PACAP inhibit both TNF-α and IL-6 expression in endotoxemic mice (9, 12, 63). Therefore, through the inhibition of proinflammatory cytokines such as TNF-α and IL-6 and the stimulation of anti-inflammatory cytokines such as IL-10, VIP/PACAP released within the lymphoid microenvironment may play an important role in the down-regulation of the inflammatory response by significantly affecting the local balance between pro- and anti-inflammatory factors. The

stimulation of the IL-10 transcription by VIP/PACAP may also have a significant therapeutical potential, since the in vivo administration of this anti-inflammatory cytokine leads to the prevention or reduction of a variety of inflammatory diseases such as endotoxic shock (53–55), viral-induced ocular inflammation (56), immune complex-mediated lung injury (57), and chronic inflammatory bowel disease (58).

Although VIP-ergic nerve fibers are found in most lymphoid organs, the respiratory and gastrointestinal tracts are particularly rich in VIP-containing fibers and cells (41, 59, 60), which release VIP upon electrical or chemical stimulation. Nitric oxide, for example, functions as a particularly potent signal for VIP release from enteric ganglia (61, 62). Therefore, nitric oxide produced during an inflammatory response could induce the release of high levels of VIP locally, especially in the respiratory and gastrointestinal tract, and the released neuropeptides could target the alveolar or peritoneal macrophages found in the immediate vicinity and down-regulate the production of proinflammatory agents.

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