

# Inhibition of Endotoxin-Induced Macrophage Chemokine Production by Vasoactive Intestinal Peptide and Pituitary Adenylate Cyclase-Activating Polypeptide In Vitro and In Vivo<sup>1</sup>

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Inflammatory chemokines recruit various populations of immune cells that initiate and maintain the inflammatory response against foreign Ags. Although such a response is necessary for the elimination of the Ag, the inflammation has to be eventually resolved in a healthy organism. Neuropeptides such as vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating polypeptide (PACAP), released after antigenic stimulation, contribute to the termination of an inflammatory response primarily by inhibiting the production of proinflammatory cytokines. Here we investigated the effects of VIP and PACAP on chemokine production. We report that VIP and PACAP inhibit the expression of the macrophage-derived CXC chemokines macrophage inflammatory protein-2 and KC (IL-8), and of the CC chemokines MIP-1 $\alpha$ , MIP-1 $\beta$ , monocyte chemoattractant protein 1, and RANTES in vivo and in vitro. The inhibition of chemokine gene expression correlates with an inhibitory effect of VIP/PACAP on NF- $\kappa$ B binding and transactivating activity. The VIP/PACAP inhibition of both chemokine production and of NF- $\kappa$ B binding and transactivating activity is mediated through the specific VIP receptor VPAC1, and involves both cAMP-dependent and -independent intracellular pathways. In an in vivo model of acute peritonitis, the inhibition of chemokine production by VIP/PACAP leads to a significant reduction in the recruitment of polymorphonuclear cells, macrophages, and lymphocytes into the peritoneal cavity. These findings support the proposed role of VIP and PACAP as key endogenous anti-inflammatory agents and describe a novel mechanism, i.e., the inhibition of the production of macrophage-derived chemokines. *The Journal of Immunology*, 2001, 167: 966–975.

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. Activated macrophages are widely recognized as cells that play a central role in the regulation of immune and inflammatory activities, as well as tissue remodeling. The execution of these activities is mediated by complex and multifactorial processes involving macrophage products. In response to Ags such as LPS, macrophages secrete proinflammatory cytokines and oxidants such as TNF- $\alpha$ , IL-6, IL-1 $\beta$ , IL-12, and NO, which contribute to pathophysiological changes associated with several acute and chronic inflammatory conditions (1), and inflammatory chemokines that recruit and activate blood-derived leukocytes (2–6). The chemokine superfamily consists of low-m.w. polypeptides that are categorized into four subfamilies, CXC, CC, C, and CX<sub>3</sub>C, based on the arrangement of positionally conserved cysteine motifs within the N terminus. The CXC and CC chemokines predominate and,

thus, have been the most extensively studied. The great interest generated by the discovery of chemokines lies in their specificity; for example, the CXC chemokines IL-8 and macrophage inflammatory protein (MIP)<sup>3</sup>-2 activate and induce the directional migration of neutrophils, whereas CC chemokines, including MIP-1 $\alpha$ , MIP-1 $\beta$ , monocyte chemoattractant protein (MCP)-1, and RANTES, are chemotactic for monocytes/macrophages and T cells (2–6).

Vasoactive intestinal peptide (VIP) and the structurally related peptide, the pituitary adenylate cyclase-activating polypeptide (PACAP), are two neuropeptides present in the immune microenvironment (7) that elicit a broad spectrum of biological functions, including actions on innate and adaptive immunity (7–9). 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 (10), and to inhibit several macrophage functions, including phagocytosis, respiratory burst, and chemotaxis (11), as well as LPS-induced IL-6, TNF- $\alpha$ , IL-12, and NO production (8, 9). In agreement with their anti-inflammatory role, VIP/PACAP recently were reported to protect mice from lethal endotoxemia, presumably by down-regulating endogenous proinflammatory macrophage-derived mediators (12).

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<sup>3</sup> Abbreviations used in this paper: MIP, macrophage inflammatory protein; MCP, monocyte chemoattractant protein; VIP, vasoactive intestinal peptide; PACAP, pituitary adenylate cyclase-activating polypeptide; PAC, PACAP receptor; VPAC, VIP receptor; db-cAMP, dibutyryl cAMP; PMN, polymorphonuclear cell(s); RPA, RNase protection assay; PKA, protein kinase A; CBP, CREB-binding protein; TBP, TATA-box binding protein.

Because chemokines are involved in controlling the nature and magnitude of the inflammatory response, in this study, we examined the *in vitro* and *in vivo* effects of VIP and PACAP on CXC and CC chemokine production in activated peritoneal macrophages, and how the VIP/PACAP regulation of chemokine expression is functionally linked to leukocyte migration. This study further clarifies 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 (PAC)1/VIP receptor (VPAC) 2 antagonist PACAP<sub>6–38</sub> was obtained from Peninsula Laboratories (Belmont, CA). The VPAC1 antagonist [Ac-His<sup>1</sup>, D-Phe<sup>2</sup>, K<sup>15</sup>, R<sup>16</sup>, L<sup>27</sup>] VIP<sub>3–7</sub>-GRF<sub>8–27</sub> and the VPAC1 agonist [K<sup>15</sup>, R<sup>16</sup>, L<sup>27</sup>] VIP<sub>1–7</sub>-GRF<sub>8–27</sub> were donated by Patrick Robberecht (Universite Libre de Bruxelles, Brussels, Belgium). The VPAC2 agonist Ro25-1553 Ac-[Glu<sup>8</sup>, Lys<sup>12</sup>, Nle<sup>17</sup>, Ala<sup>19</sup>, Asp<sup>25</sup>, Leu<sup>26</sup>, Lys<sup>27,28</sup>, Gly<sup>29,30</sup>, Thr<sup>31</sup>]-VIP cyclo<sub>21–25</sub> was a generous gift from A. Welton and D. R. Bolin (Hoffmann-La Roche, Nutley, NJ). The synthetic PAC1 agonist maxadilan was a generous gift from E. A. Lerner (Massachusetts General Hospital, Charlestown, MA). Capture and biotinylated Abs against murine KC (IL-8), RANTES, MIP-2, and MCP-1 were purchased from PeproTech (Rocky Hill, NJ). LPS (from *Escherichia coli* 055:B5), DEAE-dextran, protease inhibitors, dibutylcAMP (db-cAMP), and forskolin 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 p65, p50, and CREB were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Murine rIL-10 and neutralizing anti-IL-10 Abs were purchased from BD PharMingen (San Diego, CA).

### Preparation of peritoneal macrophages and cell cultures

Mouse peritoneal macrophages were elicited by i.p. injection of 2 ml of 4% Brewer's thioglycollate medium (Difco, Detroit, MI) into male BALB/c mice (aged 6–10 wk). Peritoneal exudate cells were obtained 72 h after injection by peritoneal lavage with 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 (Life Technologies, Rockville, MD), and were seeded in flat-bottom 96-well microtiter plates (Corning Glass, Corning, NY) at  $8 \times 10^4$  cells per well in a final volume of 200  $\mu$ l. The cells were incubated at 37°C for 2 h to adhere, and nonadherent cells were removed by repeated washing with RPMI 1640 medium. At least 96% of the adherent cells were Mac-1<sup>+</sup> macrophages.

Raw 264.7 mouse macrophage cells (American Type Culture Collection, Manassas, VA) were cultured in DMEM supplemented with 10% FCS. The cells were plated in flat-bottom 96-well microtiter plates at  $8 \times 10^4$  cells per well in a final volume of 200  $\mu$ l for 24 h, followed by two washings with DMEM medium.

Macrophage monolayers were stimulated with 10 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% CO<sub>2</sub>. Cell-free supernatants were harvested at the designated time points and kept frozen (–20°C) until chemokine determination by ELISA.

### Plasmids, transfections, and luciferase assay

NF- $\kappa$ B-dependent gene expression was evaluated with a luciferase reporter gene driven by four tandem copies of the  $\kappa$ B<sub>4</sub> in a pUC vector (Clontech, Palo Alto, CA). The plasmid pRc/RSV-p65 containing the entire cDNA of p65 was provided by G. J. Nabel and J. Stein through the National Institutes of Health AIDS Research and Reference Reagent Program. Empty vectors pRc/RSV and pUC-18 (Invitrogen, Carlsbad, CA) were used to keep constant total transfected DNA concentration in each experiment. To assess variations in transfection efficiencies, cells were transfected with 2  $\mu$ g of the control plasmid pCH110 (Amersham Pharmacia Biotech, Piscataway, NJ), which expresses the *LacZ* gene. Levels of  $\beta$ -galactosidase were determined by the Galacto-Light assay system (Tropix, Bedford, MA) and exhibited <15% variation between samples.

Raw 264.7 cells were transiently transfected with a total of 10–30  $\mu$ g of plasmid DNA by the DEAE-dextran procedure as described previously (13). Twenty-four hours after transfection, cells were stimulated with LPS (10 ng/ml) in the absence or presence of difference concentrations of VIP or PACAP as indicated above. After 6 h of incubation, luciferase assays

were conducted according to the instructions of the manufacturer (Promega, Madison, WI). Light emission was measured in a luminescence microplate counter (Top-Count; Packard, Meriden, CT). Luciferase activity, expressed in arbitrary light units, was corrected for protein concentration or normalized to coexpressed  $\beta$ -galactosidase levels.

### EMSA

Nuclear extracts were prepared as described previously (14). Double-stranded oligonucleotides (50 ng) corresponding to the NF- $\kappa$ B sites from murine MCP-1 (5'-ACTGCCCTCAGAATGGGAATTTCCACGCTCTTA TC-3'; Ref. 15), RANTES (5'-TTTTGGAACTCCCCTTAGGGGATGC CCCTCA-3'; Ref. 16), MIP-2 (5'-CCCTGAGCTCAGGGAATTTCCCT GGTCCCCG-3'; Ref. 17), and KC (5'-TACTCCGGGAATTTCCCTGG CC-3'; Ref. 18), were end-labeled with [ $\gamma$ -<sup>32</sup>P] ATP by using T4 polynucleotide kinase. For EMSAs with Raw 264.7 nuclear extracts, 20,000–50,000 cpm of double-stranded oligonucleotides, corresponding to ~0.5 ng, were used for each reaction. The binding reaction mixtures (15  $\mu$ l) were set up containing 0.5–1 ng of DNA probe, 5  $\mu$ g of nuclear extract, 2  $\mu$ g 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 mixtures were incubated on ice for 15 min before adding the probe, followed by for 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 Ab supershift experiments, the nuclear extracts were incubated for 15 min at room temperature with the specific Ab (1  $\mu$ g) or competing cold oligonucleotide (50-fold excess) before the addition of the labeled probe.

### Model of acute inflammation: LPS peritonitis

Male BALB/c mice (6–10 wk old) received a single i.p. dose of LPS (25  $\mu$ g/mouse, ~1.2–1.4 mg/kg) in the presence or absence of different amounts of VIP or PACAP (from 0.5 to 10 nmol/mouse). After different time periods (1 to 48 h), blood was removed through cardiac puncture, and peritoneal exudate was obtained by peritoneal lavage with 3 ml of ice-cold PBS containing 3 mM EDTA. The blood samples were allowed to clot for 1 h at room temperature, and serum was obtained and kept frozen until used in chemokine ELISA. The peritoneal suspension was centrifuged 5 min at 1800  $\times g$ , and cell-free supernatants (peritoneal fluid) were harvested and assayed for chemokine production. Peritoneal cells were counted and adjusted in PBS/3 mM EDTA medium at  $3 \times 10^6$  cells/ml. The number of viable cells in the different peritoneal subpopulations was determined by flow cytometry (FACScan; BD Biosciences, Mountain View, CA). Briefly, peritoneal lymphocytes, macrophages, and polymorphonuclear cells (PMN) were gated according to their different forward scatter and side scatter characteristics and counted. Alternatively, aliquots of the lavage fluid were stained with Turk's solution (0.01% crystal violet in 3% acetic acid), and differential cell counts were performed by light microscopy. The large predominance of neutrophils (99%) in the PMN population was confirmed in cytospin preparations stained with May-Grunwald and Giemsa.

### Chemokine ELISA

The content of chemokines in the culture supernatants, serum, and peritoneal fluid was determined by specific sandwich ELISAs as recommended by the manufacturer. The lower limit of detection for MCP-1, MIP-1 $\alpha$ , MIP-1 $\beta$ , MIP-2, RANTES, and KC was 16, 5, 7, 8, 10, and 15 pg/ml, respectively.

### RNase protection assay (RPA) for the detection of chemokine mRNA expression

Peritoneal macrophages were cultured at a concentration of  $2 \times 10^6$  cells/ml in 100-mm tissue culture dishes and stimulated with LPS (10 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 with the Ultraspec RNA reagent (Biotec Laboratories, Houston, TX) as recommended by the manufacturer. RPA were performed on 2.5–5  $\mu$ g of RNA with the Riboquant MultiProbe RPA system (BD PharMingen) following the manufacturer's instructions. Each commercial kit contained a set of chemokine templates as well as a template for the housekeeping gene, GAPDH. [ $\alpha$ -<sup>32</sup>P]UTP-labeled antisense RNA probes were synthesized by *in vitro* transcription from these cDNA templates. Antisense RNA probes were purified by phenol/chloroform extraction and ethanol precipitation and were hybridized with the RNA samples at 56°C overnight. Unhybridized single-stranded RNA was digested

by RNase treatment. Double-stranded RNA was purified by phenol/chloroform extraction and ethanol precipitation. The samples were electrophoresed on a 5% denaturing polyacrylamide gel. The gel then was dried and exposed to x-ray films (Kodak, Rochester, NY). Signal quantitation was performed in a PhosphorImager SI (Molecular Dynamics, Sunnyvale, CA).

## Results

### VIP and PACAP inhibit CXC and CC chemokine production by LPS-stimulated macrophages

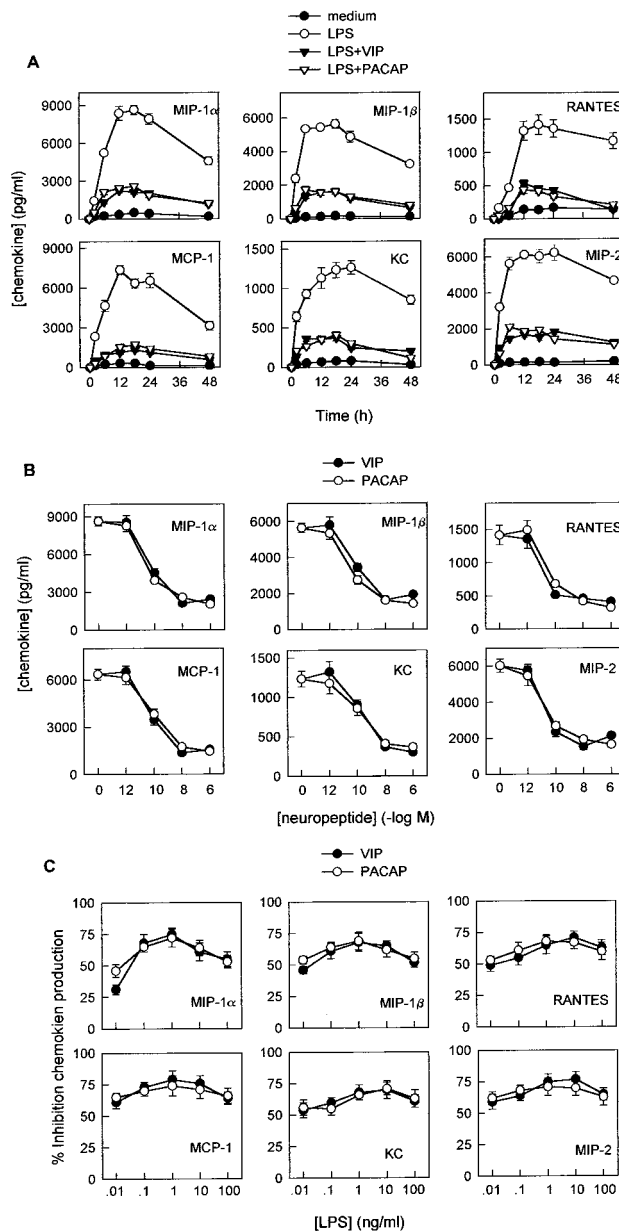
To determine the effect of VIP and PACAP on endotoxin-induced chemokine production, peritoneal macrophages were stimulated with different concentrations of LPS in the absence or presence of various doses of VIP or PACAP. The amounts of various released chemokines were assayed by ELISA. Unstimulated macrophages produce very low amounts of chemokines (Fig. 1A). Treatment with LPS results in a time-dependent increase in the production of the CXC chemokines MIP-2 and KC, as well as of the CC chemokines MIP-1 $\alpha$ , MIP-1 $\beta$ , MCP-1, and RANTES, with peak levels between 12 and 24 h (Fig. 1A). In general, CXC and CC chemokine levels remain high, with only a gradual decline between 24 and 48 h. VIP and PACAP inhibit in a dose- and time-dependent manner the production of the CXC and CC chemokines tested (Fig. 1). This inhibition occurred as early as 2 h, with a maximum effect at 24 h (Fig. 1A). Moreover, the reduction of chemokine production was maintained throughout 72 h (data not shown), indicating that VIP/PACAP do not delay, but rather reduce chemokine release. 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 concentrations, showing maximal effect at 1–10 ng/ml LPS (Fig. 1C).

The inhibitory effects were not the result of a decreased number of peritoneal macrophages, as neither VIP nor PACAP affected cell numbers or the viability of stimulated macrophages after 36 h of culture (viabilities were in the range of 91–97% with or without neuropeptides).

Because the highest inhibition was observed for macrophages stimulated with 10 ng/ml LPS, at a neuropeptide concentration of  $10^{-8}$  M after 24 h of culture, we used these conditions in the rest of the experiments.

### Inhibition of chemokine production by VIP and PACAP is mediated through VPAC1

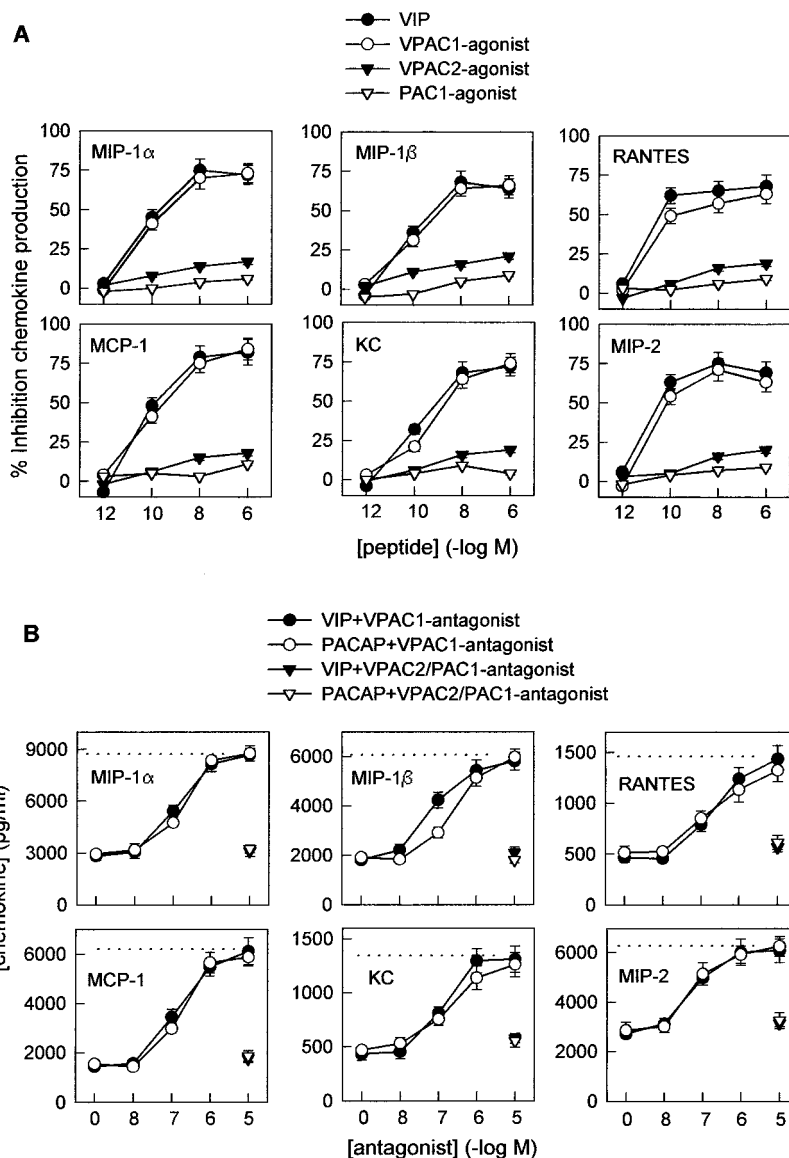
Next we investigated whether the inhibitory effect of VIP/PACAP could be related to occupancy of specific receptors. The immunological actions of VIP and PACAP are exerted through a family of receptors consisting of VPAC1, VPAC2, and PAC1 (19). Peritoneal macrophages express VPAC1 and PAC1 mRNA constitutively, and VPAC2 mRNA is inducible on LPS stimulation (20). To determine which of the VIP/PACAP receptors are involved in the inhibition of chemokine production, we used specific receptor agonists and antagonists. We investigated the effect of a VPAC1 agonist (21), a VPAC2 agonist (Ro 25-1553; Ref. 22), and of maxadilan, a specific PAC1 agonist (23) on LPS-induced chemokine production. The VPAC1 agonist, but not the VPAC2 and PAC1 agonists, inhibited the release of all CXC and CC chemokines assayed with a potency similar to that of VIP/PACAP (Fig. 2A). In addition, we investigated the ability of PACAP<sub>6–38</sub>, an antagonist specific for PAC1 and, to a lesser degree for VPAC2 (24), and of a specific VPAC1 antagonist (25), to reverse the effects of VIP and PACAP. Increasing concentrations of the antagonists ( $10^{-8}$  to  $10^{-5}$  M) were added simultaneously with a fixed concentration of



**FIGURE 1.** VIP and PACAP inhibit production of CXC and CC chemokines. **A**, Time curve for the inhibitory effect of VIP and PACAP on chemokine production. Peritoneal macrophages were stimulated with LPS (10 ng/ml) in the absence or presence of  $10^{-8}$  M VIP or PACAP, and supernatants harvested at different times were assayed for chemokine production by ELISA. **B**, Dose-response curve. Peritoneal macrophages were stimulated with LPS (10 ng/ml) and a concentration range of either VIP or PACAP for 24 h. The chemokine amounts in the culture supernatants were determined by ELISA. **C**, VIP and PACAP exert their inhibitory effect over a wide range of LPS concentrations. Peritoneal macrophages were stimulated with different concentrations of LPS in the presence or absence of  $10^{-8}$  M VIP or PACAP. After 24 h, the chemokine amounts were determined by ELISA. Percentage inhibition was calculated by comparison with controls containing LPS alone. Each result is the mean  $\pm$  SD of four separate experiments performed in duplicate.

VIP or PACAP ( $10^{-8}$  M). The VPAC1 antagonist reversed the effect of VIP/PACAP in a dose-dependent manner (Fig. 2B). In contrast, PACAP<sub>6–38</sub> did not reverse the inhibitory effect of VIP and PACAP (Fig. 2B). Together, these results suggest that VIP and PACAP exert their action primarily through VPAC1.

**FIGURE 2.** The effect of VIP/PACAP is mediated through VPAC1. **A**, Comparative effects of VPAC/PAC agonists. Peritoneal macrophages were stimulated with LPS (10 ng/ml) 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 were collected 24 h later and assayed for chemokine production by ELISA. Percentage inhibition was calculated by comparison with controls containing LPS alone. Each result is the mean  $\pm$  SD of five experiments. Each sample was assayed in duplicate. **B**, Effect of PAC1 and VPAC antagonists on the inhibitory effect of VIP and PACAP. Peritoneal macrophages were stimulated with LPS (10 ng/ml) and treated simultaneously with VIP or PACAP ( $10^{-8}$  M), and different concentrations of the VPAC1 antagonist,  $[Ac-His^1, D-Phe^1, K^{15}, R^{16}, L^{27}]VIP_{3-7}-GRF_{8-27}$ , or the PAC1/VPAC2 antagonist (PACAP<sub>6-38</sub>). Supernatants were collected 24 h later and assayed for chemokines by ELISA. The VPAC1 antagonist ( $10^{-6}$  M) and PACAP<sub>6-38</sub> ( $10^{-6}$  M) alone did not affect chemokine production in LPS-treated macrophages. 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.



#### Intracellular signal pathways involved in the inhibitory effect of VIP and PACAP on chemokine production

To determine whether intracellular cAMP mediates the inhibitory effect of VIP/PACAP on chemokine production, we determined the effects of calphostin C (a protein kinase C 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 all chemokines in LPS-stimulated macrophages, although they show less of an effect at lower concentrations (0.1 and 10 nM), compared with VIP and PACAP (Fig. 3A). The involvement of cAMP also is supported by the effects of the two protein kinase inhibitors. In contrast to calphostin C, H89 partially reverses the inhibitory effect of VIP/PACAP (Fig. 3B). These results suggest that the inhibitory effect of VIP/PACAP is partially mediated through increases in intracellular cAMP.

#### The inhibitory effects of VIP and PACAP are not mediated through IL-10

Because VIP/PACAP enhance IL-10 production in LPS-stimulated macrophages (26) and IL-10 was shown to inhibit chemokine production (27), we investigated whether the inhibitory effect of VIP/PACAP is mediated through IL-10. LPS-stimulated macrophages

treated with murine rIL-10 expressed reduced levels of MIP-1 $\alpha$ , RANTES, MCP-1, and MIP-2, and anti-IL-10 Abs (but not control Abs) enhanced chemokine production (Fig. 4). However, the anti-IL-10 Abs did not reverse the inhibitory effect of VIP or PACAP on chemokine production (Fig. 4), indicating that the effect of VIP/PACAP is not mediated through IL-10.

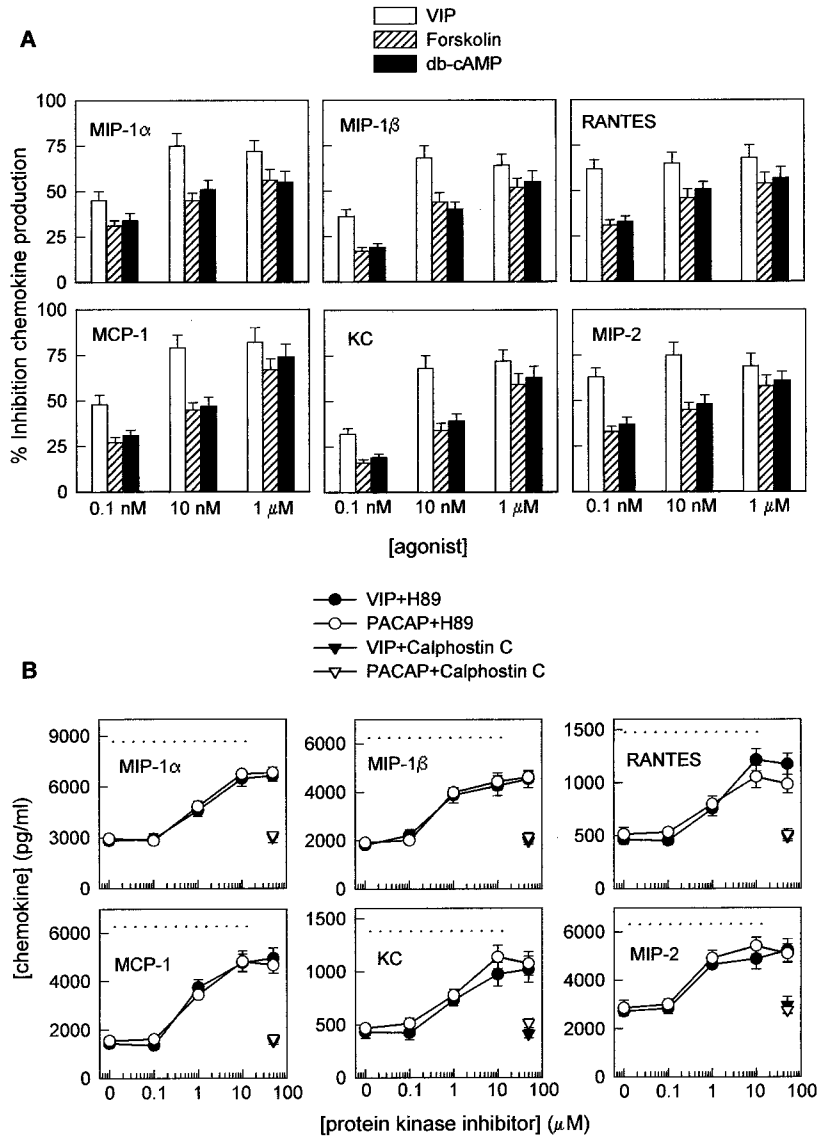
#### VIP and PACAP inhibit chemokine production at the mRNA level

Peritoneal macrophages were stimulated 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 subjected to RPA analysis. Progressively increased levels of MIP-2, MIP-1 $\alpha$ , MIP-1 $\beta$ , MCP-1, and RANTES mRNA are present in LPS-stimulated cells (up to 6–12 h; Fig. 5). At all time points, VIP and PACAP significantly inhibited the levels of both CXC and CC chemokine mRNA, with a maximum effect at 6–12 h (Fig. 5). The amount of mRNA correlated with the release of chemokine proteins in replica dishes (data not shown).

#### VIP and PACAP prevent NF- $\kappa$ B binding and subsequent NF- $\kappa$ B-dependent gene activation

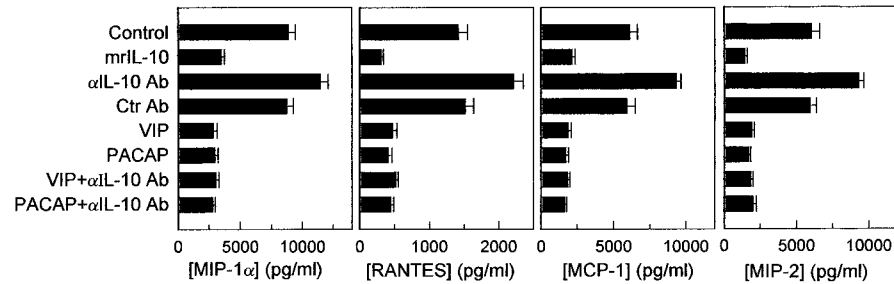
Although the promoters of most of CXC and CC chemokines contain complex arrays of transactivating binding sites, NF- $\kappa$ B

**FIGURE 3.** Intracellular signals involved in the inhibitory effect of VIP and PACAP. **A**, Effect of cAMP-inducing agents. Peritoneal macrophages were stimulated with LPS (10 ng/ml) in the presence or absence of different concentrations of VIP, forskolin, or db-cAMP. Supernatants collected after 24 h were assayed for chemokines by ELISA. Percentage of 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 protein kinase C inhibitor) and H89 (a PKA inhibitor). Peritoneal macrophages were stimulated with LPS (10 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 after 24 h were assayed for chemokines by ELISA. 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.



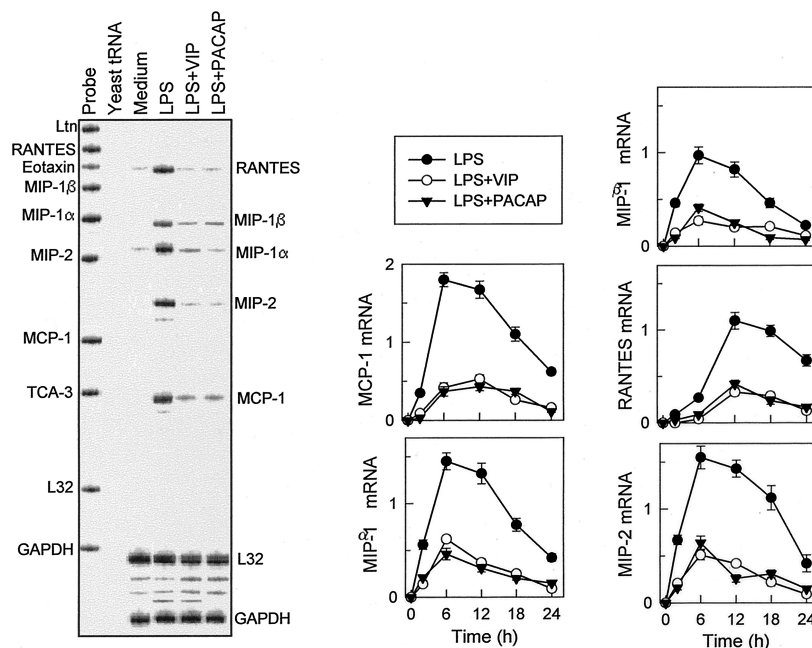
ap-pears to be essential for maximal chemokine transcription after LPS stimulation (15–18, 28–30). We have reported previously that VIP and PACAP inhibit production of several proinflammatory cytokines by activated macrophages by down-regulating NF- $\kappa$ B binding and activation (13, 31–33). To investigate whether VIP/PACAP affect NF- $\kappa$ B binding, we used nuclear extracts from LPS-stimulated peritoneal macrophages treated with or without VIP or

PACAP. VIP and PACAP inhibited the binding to  $\kappa$ B sites from the KC, MIP-2, MCP-1, and RANTES promoters (Fig. 6). The binding specificity was tested with specific ( $\kappa$ B) and nonspecific (cAMP response element) competitors, and the composition of the binding complexes (p65/p50) was determined by supershift assays (Fig. 6). Because peritoneal macrophages exhibited low transfection efficiencies, we used the murine macrophage cell line Raw



**FIGURE 4.** The inhibitory effects of VIP and PACAP on chemokine production are not mediated through IL-10. Peritoneal macrophages were stimulated with LPS (10 ng/ml), LPS plus VIP ( $10^{-8}$  M) or LPS plus PACAP ( $10^{-8}$  M) in the presence or absence of control or anti-IL-10 mAbs (10  $\mu$ g/ml). Supernatants were collected 24 h later and assayed for chemokine production. Cells treated with LPS plus IL-10 (20 ng/ml) were used in control cultures. Each result is the mean  $\pm$  SD of four experiments performed in duplicate.

**FIGURE 5.** VIP and PACAP reduce chemokine mRNA expression. Peritoneal macrophages were stimulated with LPS (10 ng/ml) in the presence or absence  $10^{-8}$  M VIP or PACAP. At different times (6 h for blot, left), cells were lysed and subjected to RPA as described in *Materials and Methods*. Results (mean  $\pm$  SD of four experiments performed in duplicate) are expressed in arbitrary densitometric units normalized for the expression of GAPDH in each sample.



264.7. First, we confirmed that VIP and PACAP affect chemokine production in the Raw cells similar to peritoneal macrophages. Indeed, VIP and PACAP inhibit in a dose- and time-dependent manner the production of KC, MIP-2, MIP-1 $\alpha$ , MIP-1 $\beta$ , MCP-1, and RANTES (Fig. 7A). Similar to peritoneal macrophages, stimulation of Raw cells with LPS led to an increase in NF- $\kappa$ B binding to the  $\kappa$ B sites from the KC, MIP-2, MCP-1, and RANTES promoters, and treatment with VIP and PACAP significantly inhibited this binding (Fig. 7B).

To determine the effects of VIP/PACAP on NF- $\kappa$ B transactivating activity, Raw 264.7 cells were transiently transfected with a ( $\kappa$ B)<sub>4</sub>-luciferase reporter plasmid, and 48 h later, the cells were

stimulated with LPS in the presence or absence of VIP or PACAP and assayed for NF- $\kappa$ B-dependent transcription 5 h later. LPS stimulation leads to an  $\sim$ 18-fold increase in NF- $\kappa$ B transcriptional activity (Fig. 7C). Treatment with VIP or PACAP strongly inhibits LPS-induced NF- $\kappa$ B activity (Fig. 7C). The inhibitory effect is dose-dependent, with a maximum at 10 nM VIP or PACAP (Fig. 7C). These effects are similar to those observed with the human monocytic cell line THP-1 (13).

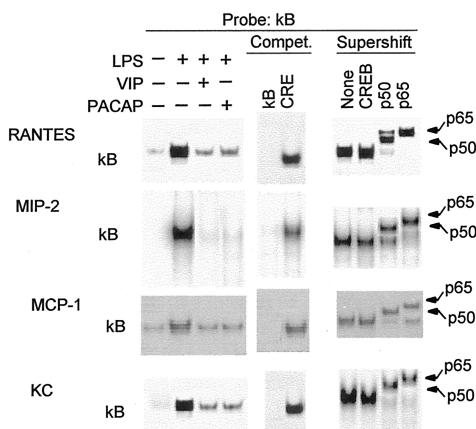
#### *Receptors and intracellular pathways involved in the inhibitory activity of VIP and PACAP on the NF- $\kappa$ B complex*

Because 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  $\kappa$ B-binding complexes and NF- $\kappa$ B-dependent gene activation. The inhibitory activity of VIP on LPS-mediated NF- $\kappa$ B binding was completely reversed by the VPAC1 antagonist (Fig. 8A, lane 3), but not by H89 (Fig. 8A, lane 4). These results suggest that the inhibition of NF- $\kappa$ B binding by VIP is mediated through VPAC1, but is mostly cAMP-independent. This is supported by the fact that forskolin (a cAMP inducer) does not significantly affect NF- $\kappa$ B binding (Fig. 8A, lane 5).

The VIP inhibition of NF- $\kappa$ B transcriptional activity is completely reversed by the VPAC1 antagonist, and partially reversed by increasing concentrations of H89 (Fig. 8B). In addition, forskolin mimics partially the effect of VIP (Fig. 8B). These findings suggest that the effect of VIP on NF- $\kappa$ B transactivating activity is mediated through VPAC1 and involves both cAMP-dependent and cAMP-independent pathways.

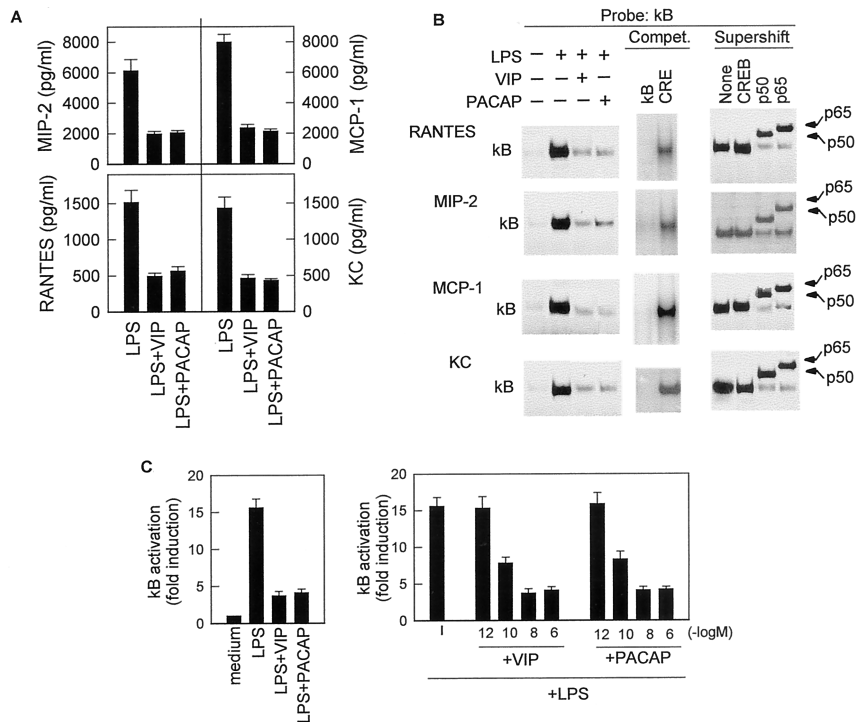
#### *In vivo inhibition of endotoxin-induced chemokine production by VIP and PACAP*

An attempt was made to reproduce the previous observations in vivo. Injection i.p. of LPS (25  $\mu$ g) resulted in the transient elevation of KC, MIP-2, MIP-1 $\alpha$ , MIP-1 $\beta$ , MCP-1, and RANTES in serum and peritoneal exudate fluid (Fig. 9). Simultaneous treatment of mice with VIP or PACAP significantly reduced the in vivo LPS-induced CXC and CC chemokines (Fig. 9). The effects of VIP and PACAP were dose-dependent, with a maximum effect at 5–10 nmol/mouse (data not shown).



**FIGURE 6.** VIP and PACAP inhibit NF- $\kappa$ B binding in peritoneal macrophages. Nuclear extracts were prepared from peritoneal macrophages incubated for 2 h with LPS (10 ng/ml) in the presence or absence of VIP or PACAP ( $10^{-8}$  M). NF- $\kappa$ B binding was assessed by EMSA with radiolabeled oligonucleotides containing the murine NF- $\kappa$ B sites from the promoters of RANTES, MIP-1 $\alpha$ , MCP-1, and KC. *Middle*, Specificity was assessed by adding a 50-fold excess of unlabeled homologous (NF- $\kappa$ B) or nonhomologous (cAMP response element) oligonucleotide to the nuclear extracts. *Right*, Identification of the proteins bound to the NF- $\kappa$ B site, by using supershift analysis. Nuclear extracts were incubated with polyclonal Abs against CREB, p65, or p50 for 20 min before adding the oligonucleotide probe. Similar results were observed in three independent experiments.

**FIGURE 7.** Effects of VIP/PACAP on chemokine production and NF- $\kappa$ B transactivating activity in Raw 264.7 cells. **A**, Raw 264.7 cells ( $8 \times 10^4$  cells/well) were cultured for 18 h with LPS (10 ng/ml) in the presence or absence of VIP or PACAP ( $10^{-8}$  M). Supernatants were assayed for RANTES, MIP-2, KC, and MCP-1 concentrations by ELISA. **B**, Nuclear extracts were prepared from Raw 264.7 cells incubated for 2 h with LPS (10 ng/ml) in the presence or absence of VIP or PACAP ( $10^{-8}$  M). NF- $\kappa$ B binding was assessed by EMSA as in Fig. 6. Similar results were observed in three independent experiments. **C**, VIP and PACAP inhibit LPS-induced NF- $\kappa$ B transactivating activity. Raw 264.7 cells were transiently transfected with the  $(\kappa B)_4$ -Luc construct (10  $\mu$ g) by the DEAE-dextran method. Forty-eight hours after transfection, cells were incubated with medium alone or stimulated with LPS (500 ng/ml) in the absence or presence of different concentrations of VIP or PACAP ( $10^{-8}$  M; *left*) for 6 h. Cytosolic extracts (100  $\mu$ g) were used in luciferase assays. Fold induction is relative to luciferase activity in unstimulated cells. Data are expressed as the mean  $\pm$  SD of three independent experiments performed in duplicate.

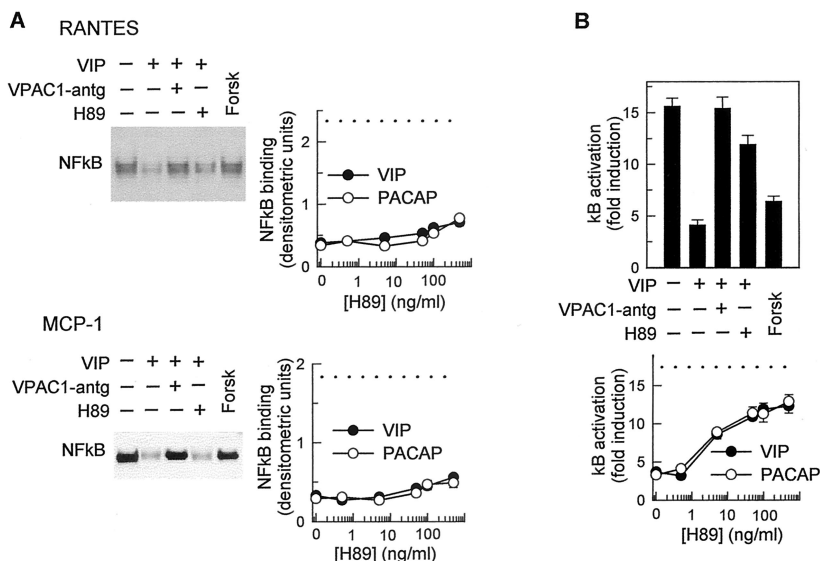


#### VIP and PACAP prevent recruitment of leukocytes within the peritoneal cavity in an inflammatory model of acute peritonitis

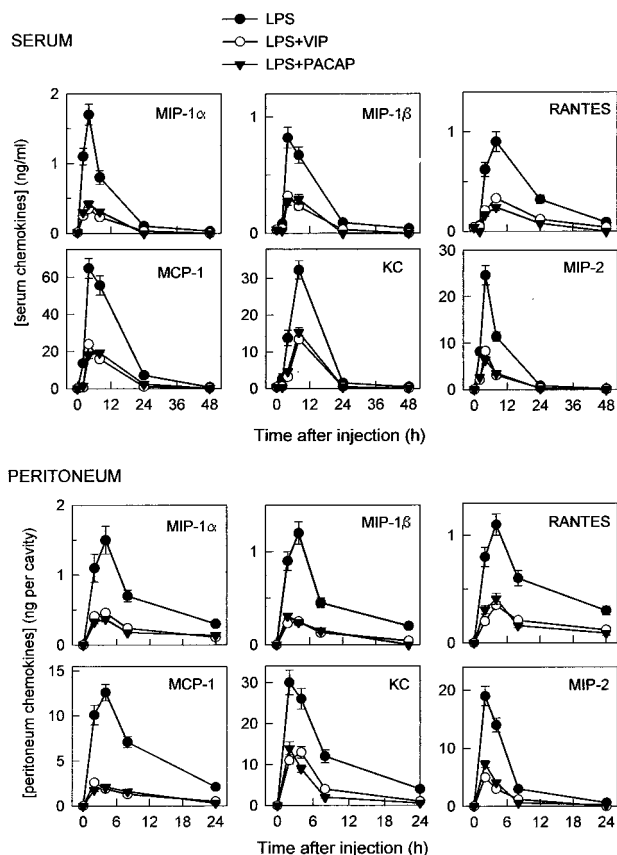
Chemokines are chemoattractants for blood-derived leukocytes, and there is a clear link between chemokine production and leukocyte recruitment in acute inflammatory models (2–6). We investigated whether VIP and PACAP also are capable of inhibiting leukocyte recruitment in a murine model of acute inflammation. Injection i.p. of LPS induces a time-dependent accumulation in the peritoneal cavity of various cell populations. PMN, predominantly neutrophils, accumulate first, with a maximal influx at 12 h after endotoxin administration (Fig. 10). Macrophages and lymphocytes are slightly delayed, starting to accumulate between 12 and 18 h, peaking at 24 h, and being still significantly elevated at 48 h (Fig. 10). Treatment with VIP or PACAP (5 nmol/mouse) significantly reduced LPS-induced PMN, macrophage, and lymphocyte influx into the peritoneal cavity (Fig. 10).

#### Discussion

Human septic shock syndrome, a systemic response to severe bacterial infections, is initiated by Gram-negative bacterial endotoxins (34, 35). Administration of LPS, an integral outer membrane component of Gram-negative bacteria, in experimental animals leads to pathophysiological changes similar to the human septic shock syndrome. Exposure to bacterial endotoxins initiates a rapid, coordinated recruitment and activation of neutrophils, monocytes/macrophages, and T cells (2–4), followed by overproduction of proinflammatory mediators, leading to tissue damage that precedes multiple organ failure. Similar to anti-inflammatory cytokines such as IL-10 and IL-13 (36, 37), exogenous administration of VIP or PACAP protects mice from the lethal effect of high endotoxemia, presumably by down-regulating the production of proinflammatory mediators such as IL-6, TNF- $\alpha$ , IL-12, NO, and IFN- $\gamma$  (12).



**FIGURE 8.** Specific receptors and intracellular pathways involved in the VIP and PACAP regulation of NF- $\kappa$ B binding and transactivating activity. Raw 264.7 cells were activated with LPS (500 ng/ml) in the absence (lane 1) or presence of VIP ( $10^{-8}$  M; lane 2) or forskolin ( $10^{-6}$  M; lane 5). VPAC1 antagonist ( $10^{-7}$  M; lane 3) or different concentrations of H89 (100 ng/ml; lane 4) were added simultaneously with VIP ( $10^{-8}$  M). **A**, NF- $\kappa$ B binding was analyzed 1 h after stimulation by EMSA as described in Fig. 5. For blots, one representative experiment of three is shown. Results shown in graphs are the mean  $\pm$  SD of three independent experiments performed in duplicate. Dotted lines in graphs of A and B represent control values of samples treated with LPS alone.

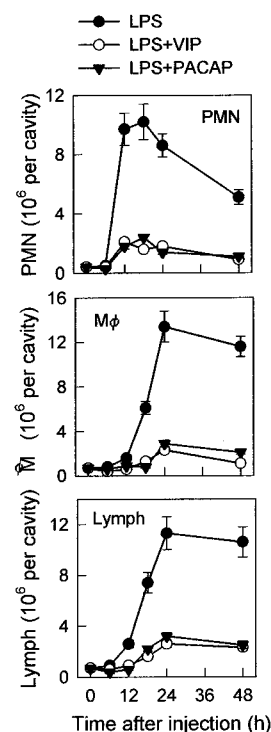


**FIGURE 9.** In vivo effects of VIP and PACAP on chemokine production. Mice (groups of four) were injected i.p. with LPS (25  $\mu$ g/mouse), LPS plus VIP (5 nmol/mouse), or LPS plus PACAP (5 nmol/mouse). Serum and peritoneal fluid were obtained at the indicated time points as described in *Materials and Methods* and were assayed for chemokines by ELISA. Each result is the mean  $\pm$  SD of three experiments.

The present study demonstrates a novel property of VIP/PACAP that might contribute to their anti-inflammatory effects. VIP and PACAP inhibit the production of the proinflammatory CXC chemokines KC and MIP-2, and of the CC chemokines MIP-1 $\alpha$ , MIP-1 $\beta$ , MCP-1, and RANTES by LPS-stimulated macrophages. The inhibitory effect is dose-dependent within a wide range of neuropeptide concentrations ( $10^{-6}$ – $10^{-10}$  M), with a maximum effect at  $10^{-8}$  M. This is the dose range in which VIP and PACAP modulate several other immunological functions (8–11).

Because the anti-inflammatory action of VIP and PACAP is mediated primarily through a direct effect on macrophages (8, 9), and because LPS-stimulated macrophages elicit the proinflammatory cascade associated with sepsis (34, 35), we focused on chemokine production by LPS-stimulated peritoneal macrophages.

Both peritoneal macrophages and the Raw 264.7 cell line express VPAC1 and PAC1 mRNA constitutively and VPAC2 mRNA after LPS-stimulation (20, 31). 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 CK production also is supported by the fact that a VPAC1 antagonist, but not PACAP<sub>6–38</sub>, an antagonist specific for PAC1 and to a lesser degree for VPAC2, reverses the inhibitory effect of VIP/PACAP. Also, the VPAC1 antagonist blocked the effect of VIP/PACAP on NF- $\kappa$ B binding to the  $\kappa$ B site specific for the KC, RANTES, MIP-1 $\alpha$  and MCP-1 promoters, supporting the involvement of VPAC1 in the inhibitory effect of VIP/PACAP on chemokine gene expression.



**FIGURE 10.** VIP and PACAP prevent in vivo leukocyte recruitment in the peritoneal cavity. Mice (groups of five) were injected i.p. with LPS (25  $\mu$ g/mouse), LPS plus VIP (5 nmol/mouse), or LPS plus PACAP (5 nmol/mouse). At different times, peritoneal cell suspensions were collected, and numbers of PMN, macrophages (M $\phi$ ), and lymphocytes (Lymph) were determined by flow cytometry as described in *Materials and Methods*. Data are the mean  $\pm$  SD of three experiments.

The VPAC1 is coupled primarily to the adenylate cyclase system (19), and production of some chemokines (i.e., MCP-3) is indeed inhibited by agents that increase intracellular cAMP levels (38). In the present study, forskolin and PGE<sub>2</sub>, two cAMP inducing agents, 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. However, because reversal was incomplete, a second cAMP-independent pathway may participate in the transduction of the VIP/PACAP signal. Similar observations were made previously for the inhibitory effect of VIP/PACAP on TNF- $\alpha$ , IL-12, NO production in macrophages (20, 31–33), and on IL-2 and IL-10 production in lymphocytes (39). The existence of a second, cAMP-independent pathway, is supported also by the fact that at concentrations that are physiologically relevant for VIP ( $10^{-8}$  M) the peptide induces less cAMP than forskolin (32) while acting as a more potent chemokine inhibitor. The nature of this second transduction pathway remains to be determined.

Chemokine synthesis is controlled at several levels. Whereas posttranscriptional, translational, and posttranslational mechanisms play important roles, chemokine transcription appears to be the primary regulatory site. The present study indicates that the inhibitory effect of VIP and PACAP on CXC and CC chemokine production occurs through reduction in mRNA levels. How can VIP and PACAP regulate such a wide spectrum of chemokine genes? The answer is probably that gene expression for many chemokines depends on the pleiotropic transcription factor NF- $\kappa$ B (15–18, 28–30). NF- $\kappa$ B consists mostly of p50/p65 heterodimers which are complexed to the inhibitor I $\kappa$ B in the cytoplasm of unstimulated cells; stimuli such as LPS and proinflammatory cytokines induce the phosphorylation and degradation of I $\kappa$ B, followed

by the release and subsequent nuclear translocation of the p50/p65 heterodimers, which bind to regulatory sequences in a variety of target genes (40). The present study indicates that VIP and PACAP inhibit LPS-induced NF- $\kappa$ B/DNA binding to the  $\kappa$ B motifs in the promoters of KC, RANTES, MCP-1, and MIP-1 $\alpha$ . In addition, VIP/PACAP reduce NF- $\kappa$ B transactivating activity. The effect on NF- $\kappa$ B/DNA binding is cAMP-independent, whereas the inhibition of the NF- $\kappa$ B transactivating activity is mediated through both a cAMP-dependent and -independent pathway. This is in agreement with previous observations (13, 31–33), and is presumably attributable to the fact that NF- $\kappa$ B transactivation involves several steps in addition to NF- $\kappa$ B DNA binding. In LPS-stimulated human monocytes, we showed that VIP/PACAP act at multiple levels (13). First, VIP/PACAP inhibit p65 nuclear translocation and NF- $\kappa$ B DNA binding by stabilizing the inhibitor I $\kappa$ B $\alpha$  in a cAMP-independent manner. Second, VIP/PACAP induce the phosphorylation of CREB, which binds the coactivator CREB-binding protein (CBP), found in limiting amounts in the nucleus. This results in a decrease in p65/CBP complexes, which further reduces NF- $\kappa$ B transactivation. Third, VIP and PACAP inhibit the mitogen-activated protein/extracellular signal-related kinase kinase1/mitogen-activated protein/extracellular signal-related kinase kinase 3/6/p38 pathway, ultimately affecting the phosphorylation of the coactivator, TATA-box binding protein (TBP), resulting in a reduction in TBP binding to both p65 and the TATA box. In contrast to the effect on p65 nuclear translocation, the effects on CBP and TBP are mediated through the cAMP/PKA pathway (13).

IL-10 was reported to inhibit cytokine and chemokine production in alveolar macrophages and microglia by preventing NF- $\kappa$ B nuclear translocation (27, 41, 42). We showed previously that VIP and PACAP enhance IL-10 production in LPS-stimulated peritoneal macrophages (26). Therefore, the effects of VIP/PACAP on chemokine expression might be mediated through IL-10. However, this doesn't seem to be the case, because the addition of neutralizing anti-IL-10 Abs did not reverse the inhibitory effect of VIP/PACAP.

Because the release of chemokines results in the recruitment of blood-derived leukocytes (2–6), the fact that VIP and PACAP inhibit chemokine production in vivo is of obvious biological significance. In agreement with previous reports (43, 44), we found that i.p. administration of LPS results in a rapid production of chemokines. KC and MIP-2 both exhibited early (2 h) peak levels in peritoneum, consistent with their role in the recruitment of neutrophils, the first leukocyte population to arrive at a site of inflammation. Chemokines responsible for recruiting subsequent leukocyte infiltrates, i.e., monocytes/macrophages and T lymphocytes (i.e., MCP-1, MIP-1 $\alpha$ , RANTES, and MIP-1 $\beta$ ), peaked at a later time point (4 h). VIP and PACAP prevented the influx and accumulation into the peritoneal cavity of neutrophils, macrophages, and lymphocytes, presumably through the inhibition of chemokine production. These findings support the proposed role for VIP and PACAP as key anti-inflammatory agents in vivo, and suggest an additional molecular mechanism, i.e., the inhibition of macrophage-derived chemokine production.

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