Vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide modulate endotoxin-induced IL-6 production by murine peritoneal macrophages

Carmen Martínez,* Mario Delgado,* David Pozo,† Javier Leceta,* Juan R. Calvo,† Doina Ganea,‡ and Rosa P. Gomariz*

**Department of Cellular Biology, Faculty of Biology, Complutense University, Madrid, Spain; †Department of Medical Biochemistry and Molecular Biology, University of Seville, School of Medicine and Virgen Macarena Hospital, Seville, Spain; and ‡Department of Biological Sciences, Rutgers University, Newark, New Jersey*

Abstract: Vasoactive intestinal peptide (VIP) is a neuropeptide synthesized by immune cells that can modulate several immune aspects, including the function of cells involved in the inflammatory response, such as macrophages and monocytes. Production and release of cytokines by activated mononuclear phagocytes is an important event in the pathogenesis of ischemia-reperfusion injury. VIP has been shown to attenuate the deleterious consequences of this pathologic phenomenon. We have investigated the effects of VIP and PACAP38 on the production of interleukin-6 (IL-6), a pro-inflammatory cytokine, by endotoxin-activated murine macrophages. Both neuropeptides exhibit a dual effect on the IL-6 production by peritoneal macrophages. Whereas VIP and PACAP inhibit with similar dose-response curves the release of IL-6 from macrophages stimulated with a LPS dose range from 100 pg/mL to 10 μg/mL, both neuropeptides enhance IL-6 secretion in unstimulated macrophages and in macrophages stimulated with very low LPS concentrations (1–10 pg/mL). The inhibition on LPS-induced IL-6 production is specific, presumably mediated through a subtype of the PACAP-R. VIP and PACAP regulate the production of IL-6 at a transcriptional level. These results were correlated with an inhibition on both IL-6 expression and release in endotoxemic mice in vivo. These findings support the idea that in the absence of stimulation or in the presence of low doses of LPS, VIP and PACAP could play a role in immune system homeostasis. However, under toxicity conditions associated with high LPS doses, VIP and PACAP could act as protective mediators that regulate the excessive release of IL-6 in order to reduce inflammation or shock. *J. Leukoc. Biol.* 63: 591–601; 1998.

Key Words: *lipopolysaccharide · neuropeptides · cytokines*

INTRODUCTION

Vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase activating polypeptide (PACAP) are 28- and 38-amino acid peptides that have 65% homology and perform a broad spectrum of biological functions, including action on natural and acquired immunity [1, 2]. VIP is produced by lymphocytes in central and peripheral lymphoid organs [3, 4] and PACAP-positive cells appear to be present in different lymphoid organs [5]. Both VIP and PACAP exert their diverse biological action through three types of PACAP/VIP receptors recently cloned. The PACAP receptor (PACAP-R) binds the two amidated forms of PACAP, PACAP27 and PACAP38, with similar affinity, but has a 300- to 1000-fold lower affinity for VIP [6]. This receptor is coupled to the activation of both adenylate cyclase and phospholipase C (PLC) systems [6]. The type 1 VIP receptor (VIP_1/PACAP-R), which corresponds to the classical VIP receptor, recognizes, with comparable high affinity, VIP and both PACAP forms and is coupled to adenylate cyclase [6, 7]. Finally, the type 2 VIP receptor (VIP_2/PACAP-R), has similar affinities for PACAP and VIP and is coupled to the adenylate cyclase system [6, 7] and to a calcium chloride channel in transfected cells.

The immunomodulatory activities of VIP and PACAP are mediated, at least partly, through the effects on the production of cytokines. VIP was reported to stimulate interleukin-5 (IL-5) release from schistosome granulomas and splenic T lymphocytes [8], and to inhibit the expression and secretion of IL-2, IL-4, and IL-10 in stimulated peripheral T cell and thymocyte

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Abbreviations: VIP, vasoactive intestinal peptide; PACAP, pituitary adenylate cyclase-activating polypeptide; LPS, lipopolysaccharide; PACAP-R, type 1 PACAP receptor; VIP_1/PACAP-R, type 1 VIP receptor; VIP_2/PACAP-R, type 2 VIP receptor; IL, interleukin; PLC, phospholipase C; TNF-α, tumor necrosis factor α; CSPD, chemiluminescent substrate for alkaline phosphatase; FCS, fetal calf serum; ELISA, enzyme-linked immunosorbent assay; PKC, protein kinase C; PKA, protein kinase A; PBS, phosphate-buffered saline; SSC, saline sodium citrate.

Correspondence: Dr. Rosa P. Gomariz, Department of Cellular Biology, Faculty of Biology, Complutense University, Madrid, Spain. E-mail: Gomariz@eucmax.sim.ucm.es

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cultures [9–12]. In addition, we have recently found that VIP and PACAP stimulate the IL-6 production in unstimulated peritoneal macrophages [unpublished results]. IL-6 is a multifunctional cytokine that is produced by both lymphoid and nonlymphoid cells and regulates several immune responses, acute-phase reactions, and haematopoiesis, and participates as a mediator in the inflammatory response [13]. The production of IL-6 is induced by several factors, including tumor necrosis factor α (TNF-α), IL-1β, as well as the bacterial endotoxin lipopolysaccharide (LPS). LPS, a major component of the outer membrane of Gram-negative bacteria, is a potent activator of the macrophage response [14], and evokes drastic biological responses within host cells, including fever, procoagulant activity, septic shock, and death. It is well known that most endotoxic effects are mediated through the activation of the host immune and inflammatory cells, particularly macrophages that play a central role in endotoxicity through the production of factors, including nitrogen intermediates, prostaglandins, and cytokines such as TNF-α, IL-1, and IL-6, both in vivo and in vitro [13]. In this context, and because VIP has been involved in the local regulation of the inflammatory response, our purpose was to determine the effect of VIP and PACAP on the production of IL-6 in peritoneal macrophages in vivo and in vitro. We found that both neuropeptides exhibit a dual effect on IL-6 production, and we discuss their role in inflammation.

MATERIALS AND METHODS

Animals

Female Swiss-Webster mice, 6–10 weeks of age, were purchased from Iffa Credo (France). Mice were kept in the animal house for at least 1 week before use in a temperature-controlled room with a 12-h light-dark cycle and were allowed free access to standard laboratory chow and water.

Antibodies and reagents

Synthetic VIP, PACAP38, PACAP27, $VIP_{1–12}$, and $VIP_{10–28}$ were purchased from Novabiochem (Läufelfingen, Switzerland). The VIP-antagonist [*N*-Ac-Tyr^1, D-Phe^2]-GRF(1–29) amide was obtained from Cambridge Research BioChemicals (Wilmington, DE), and PACAP(6–38) (a potent PACAP38 antagonist), secretin, and glucagon were obtained from Peninsula Laboratories (Belmont, CA). The [K^{15},R^{16},L^{27}]VIP(1–7)-GRF(8–27) VIP_1/PACAP-R agonist and the [Ac-His^1,D-Phe^2,K^{15},R^{16},L^{27}]VIP(3–7)-GRF(8–27) VIP_1/PACAP-R antagonist were generous gifts from Dr. Patrick Robberecht (Universite Libre de Bruxelles). Oligonucleotides were synthesized by Pharmacia Biotech (Uppsala, Sweden). The blocking reagent, digoxigenin oligonucleotide tailing kit, anti-digoxigenin-AP (Fab fragments), and the chemoluminescent substrate for alkaline phosphatase (CSPD) were from Boehringer-Mannheim (Mannheim, Germany). Murine recombinant (mr) IL-1β, mrTNF-α, mrIL-6, and capture and biotinylated monoclonal anti-murine IL-6 antibodies were purchased from PharMingen (San Diego, CA). LPS (from *Escherichia coli* 0111:B4 and *S. enteridis*), 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS substrate), *N*-laurylsarcosyl, malic acid, sodium dodecyl sulfate, Kodak X-OMAT AR film, avidin-peroxidase, the H89, and calphostin C were purchased from Sigma Chemicals (St. Louis, MO).

Preparation of macrophages and cell cultures

Each animal was killed by cervical dislocation according to the guidelines of the European Community Council Directives 86/6091 EEC. The abdomen was cleaned with 70% ethanol, the abdominal skin carefully dissected without opening the peritoneum, and 4 mL of RPMI 1640 medium was injected intraperitoneally. Then the abdomen was massaged and about 90% of the solution, containing lymphocytes and macrophages, was extracted. Peritoneal exudate cells were washed and resuspended in ice-cold RPMI 1640 medium (GIBCO-BRL, Grand Island, NY) supplemented with 2% heat-inactivated fetal calf serum (FCS; GIBCO-BRL) containing 0.01 M *N*-2-hydroxyethylpiperazine—*N*′-2-ethanesulfonic acid buffer, 1 mM pyruvate, 0.1 M nonessential amino acids, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, 100 U/mL penicillin, and 100 μg/mL streptomycin (RPMI 1640 complete medium). Cells were plated in 24-well tissue culture plates (Corning Glass, Corning, NY) at 5×10^5 cells per well in a final volume of 1 mL. The cells were incubated at 37°C for 2 h to adhere 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 morphological and phagocytic criteria and by flow cytometry. Macrophage monolayers were incubated in RPMI 1640 complete medium and stimulated with different concentrations of LPS (from 1 pg/mL to 10 μg/mL) in the presence or absence of VIP or PACAP38 (from 10^{-14} to 10^{-6} M) at 37°C in a humidified incubator with 5% CO_2. In some experiments, macrophages were stimulated with TNF-α (10 ng/mL) or IL-1β (100 U/mL). Cell-free supernatants were harvested at the designated time points and kept frozen (−20°C) until assayed for IL-6 production by enzyme-linked immunosorbent assay (ELISA). When indicated, to determine the effect of the VIP- and PACAP-antagonists, VIP_1/PACAP-R agonist, and protein kinase C (PKC) and protein kinase A (PKA) inhibitors, cells were incubated with a concentration range of these agents, as indicated in the text, and simultaneously incubated with VIP/PACAP and LPS as described above.

Cytokine determination: ELISA assay for IL-6

The amount of IL-6 present in supernatants was determined by using a murine IL-6 capture ELISA assay essentially as described previously [15]. Briefly, a capture monoclonal anti-murine IL-6 antibody (clone MP5-20F3) was used to coat microtiter plates (Corning ELISA plates) at 2 μg/mL at 4°C for 16 h. After washing and blocking with phosphate-buffered saline (PBS) containing 3% bovine serum albumin, culture supernatants were added to each well for 12 h at 4°C. Unbound material was washed off and a biotinylated monoclonal anti-mouse IL-6 antibody (clone MP5-32C11) was added at 2 μg/mL for 2 h. Bound antibody was detected by addition of avidin-peroxidase for 30 min followed by addition of the ABTS substrate solution. Absorbances at 405 nm were taken 30 min after the addition of the substrate. A standard curve was constructed using various dilutions of murine rIL-6 in PBS containing 10% FCS. The amount of IL-6 in the culture supernatants was determined by extrapolation of absorbances to the standard curve. The ELISA assay was specific for murine IL-6 (did not cross-react with human or rat IL-6, or with other murine cytokines such as IL-10, TNF-α, IL-2, IL-3, IL-4, IL-5, IL-1α, IL-1β, and IFN-γ). The sensitivity of the assay was 10 pg IL-6/mL.

RNA isolation and Northern blot analysis

Northern blot analysis was performed according to standard methods. Peritoneal macrophage monolayers (2×10^6 cells/mL) were stimulated with LPS (10 ng/mL) in the absence or presence of VIP or PACAP38 (10^{-8} M) for 3 h at 37°C, and total RNA was isolated using the Ultraspec RNA Isolation System (Biotecx Laboratories, Houston, TX) according to the manufacturer's instructions. Briefly, macrophage monolayers (2×10^7 cells) were lysed in Ultraspec RNA reagent, followed by chloroform extraction and isopropanol precipitation. The RNA was resuspended in DEPC-treated water and quantitated spectrophotometrically at 260/280 nm. Twenty micrograms of total RNA from each sample were electrophoresed on 1.2% agarose-formaldehyde gels, transferred to S & S Nytran membranes (Schleicher and Schuell, Keene, NH), and cross-linked to the nylon membrane using UV light (Linus, Cultek SL, Spain).

The probe for IL-6 was an oligonucleotide (5′-CAAGAAGGCAACTGGATG AAGTCTCTTGCAGAGAGGAACTTCAT-3′) designed from the murine 1.078-kb mRNA for IL-6 [16]. The probe for the murine 18S rRNA, as a quantity control for RNA, was an oligonucleotide (5′-CCAATTACAGGGCCTC-GAAAGAGTCCTGTA-3′) derived from the published sequence. Oligonucleotides were 3′-labeled with digoxigenin-dUTP/dATP mix using terminal transferase according to manufacturer's instructions. After UV cross-linking the blots were washed twice for 5 min in DEPC-water and prehybridized at 68°C for 3 h in prehybridization buffer containing 5 × saline sodium citrate (SSC), 1% blocking reagent (w/v), 0.1% *N*-laurylsarcosyl (w/v), and 0.02% sodium dodecyl sulfate (SDS; w/v). The hybridization was performed at 60°C for 16 h in the same prehybridization buffer plus 25 ng/mL of IL-6 3′-labeled oligonucleo-

tide. Thereafter, the blots were washed twice for 5 min each in 2 × SSC/0.1% SDS (w/v) at room temperature, followed by washing twice for 5 min each to a final stringency of 0.1 × SSC/0.1% SDS (w/v) at 60°C. To detect the hybridization signal, the blots were incubated for 30 min in 0.1 M maleic acid (pH 7.5), 0.15 M NaCl, rinsed in 0.1 M Tris-HCl (pH 9.5), 0.1 M NaCl, and incubated in the same buffer containing 0.25 mM CSPD. The chemiluminescent signals were obtained by using Kodak X-OMAT AR film at room temperature. The membranes were stripped twice for 30 min each in Tris-HCl (pH 8.0) containing 5% *N,N*-dimethyl-formamide (v/v), 1% SDS (w/v) at 68°C, and rehybridized with the 18S rRNA-labeled antisense oligonucleotide at 55°C.

In vivo quantitation of IL-6 levels

Female mice (6- to 10-week old) were randomized to receive a single intraperitoneal dose of LPS (25 μg/mouse), LPS plus VIP (5 nmol/mouse), or LPS plus PACAP38 (5 nmol/mouse). After 2 and 3 h blood was removed through cardiac puncture and peritoneal exudate was obtained as described above. The blood samples were allowed to clot for 1 h at room temperature and serum was obtained and kept frozen until IL-6 ELISA analysis. The peritoneal suspension was centrifuged 5 min at 1800 *g* and the cell-free supernatant was harvested and assayed for IL-6 ELISA. The peritoneal cells were subjected to Northern blot analysis as described above.

Statistical analysis

All values are expressed as the mean ± SD of the number of experiments performed in duplicate, as indicated in the corresponding tables and figures. Comparisons between groups were made using the Student's *t* test followed by Scheffe's *F*-test, with $P < 0.05$ as the minimum significant level.

RESULTS

Effects of VIP and PACAP on LPS-induced IL-6 production

Previously we observed that both VIP and PACAP stimulate IL-6 production in unstimulated peritoneal macrophages [unpublished results]; this has been confirmed here (**Fig. 1A**). To determine the effects of these neuropeptides on endotoxin-induced IL-6 production, the macrophage cultures were stimulated with LPS and supernatants were harvested at different time points and assayed for IL-6 production by ELISA.

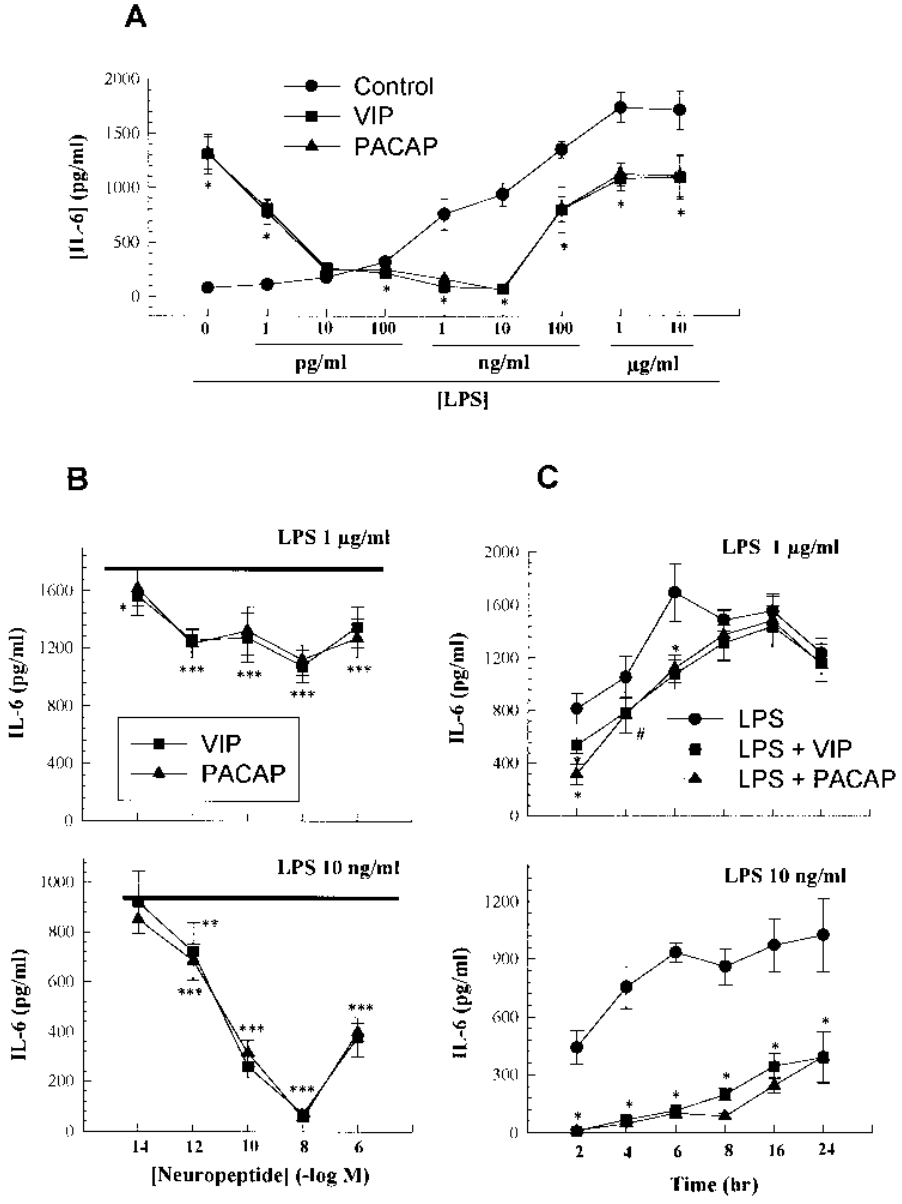


Fig. 1. Effect of VIP and PACAP on IL-6 production by LPS-stimulated murine peritoneal macrophages. (A) Peritoneal macrophages (5×10^5 cells/mL) were stimulated with LPS (1 pg/mL to 10 μg/mL) in the absence or presence of 10^{-8} M VIP or PACAP38. After a 6-h incubation period, the supernatants were collected and assayed for IL-6 production by ELISA. (B) Dose-response curve for the inhibitory effect of VIP and PACAP on LPS-stimulated IL-6 production. Peritoneal macrophages (5×10^5 cells/mL) were stimulated with LPS (10 ng/mL or 1 μg/mL) in the absence or presence of different concentrations of VIP or PACAP (from 10^{-6} to 10^{-14} M). Supernatants were collected 6 h later and assayed for IL-6 production by ELISA as described in Materials and Methods. Solid horizontal lines represent control values from cultures incubated with LPS alone (936 ± 107 pg/mL for 10 ng/mL LPS; and 1732 ± 139 pg/mL for 1 μg/mL LPS). (C) Time curve for the inhibition of LPS-induced IL-6 production by VIP and PACAP. Peritoneal macrophages (5×10^5 cells/mL) were stimulated with LPS alone (10 ng/mL and 1 μg/mL), LPS plus 10^{-8} M VIP or LPS plus 10^{-8} M PACAP, and supernatants collected at different times were assayed for IL-6 production by ELISA as described in Materials and Methods. Each result is the mean ± SD of six separate experiments performed in duplicate. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ with respect to control cultures with LPS alone.

TABLE 1. Inhibitory Effects of VIP and PACAP38 on IL-6 Release by Peritoneal Macrophages Stimulated with LPS[a]

Treatment	Maximum inhibition (%)[b]	IC_{50} (pM)[c]
VIP	88.0 ± 3.4	8.66 ± 0.68
PACAP	88.5 ± 4.5	8.66 ± 1.18

[a] Peritoneal macrophages (0.5×10^6 cells/mL) were stimulated with LPS (10 ng/mL) in the absence (control) or presence of VIP or PACAP (10^{-6}–10^{-14} M). The supernatants were harvested at 6 h and IL-6 levels were determined by ELISA. Control IL-6 levels were 902 ± 37 pg/mL.

[b] Percentage inhibition was calculated by comparing each experimental culture with its control.

[c] IC_{50}, which is the concentration of neuropeptide producing 50% of maximal inhibition, was determined by extrapolation from the dose-response curves obtained in four separate experiments.

Data are expressed as mean ± SD of four experiments performed in duplicate.

Stimulation of macrophages with LPS for 6 h resulted in a dose-dependent release of IL-6 (Fig. 1A). VIP/PACAP had a dual effect (Fig. 1A). At low LPS concentrations (1–10 pg/mL), VIP and PACAP were stimulatory, whereas at LPS concentrations higher than 100 pg/mL, both neuropeptides significantly inhibited the production of IL-6 (Fig. 1, A and B). The dose-response curves were similar for VIP and PACAP (Fig. 1B), showing maximal effects at 10^{-8} M. The inhibitory effect was dependent on the LPS concentration used, with the strongest inhibition for 10 ng/mL LPS (Fig. 1, A and B). For 10 ng/mL LPS, the inhibitory activity of VIP and PACAP reached values of 88% (**Table 1**), showing an IC_{50} (i.e., the concentration of neuropeptide producing 50% of maximal inhibition) of 8.6 pM.

Figure 1C shows the time curves for the inhibitory effect of VIP and PACAP. The IL-6 release was significantly inhibited by VIP and PACAP as early as 2 h, and the degree of inhibition was dependent on the LPS concentration (Fig. 1C). To determine whether the effect of VIP and PACAP was sustained over a longer period of time, macrophages were stimulated with LPS in the presence of VIP/PACAP during a 24-h incubation period. The reduction of IL-6 release was maintained throughout the 24-h incubation period, indicating that VIP and PACAP do not delay, but rather attenuate the IL-6 release (Fig. 1C).

Because the highest inhibition of IL-6 production was obtained with macrophages stimulated with 10 ng/mL LPS at a neuropeptide concentration of 10^{-8} M, after 6 h of culture, we used these conditions in the rest of the experiments.

The inhibitory effects were not the result of a decrease in the number of peritoneal macrophages because neither VIP nor PACAP affected cell numbers or viability (viabilities at 8 h of culture were in the range of 85 to 95% with or without peptides).

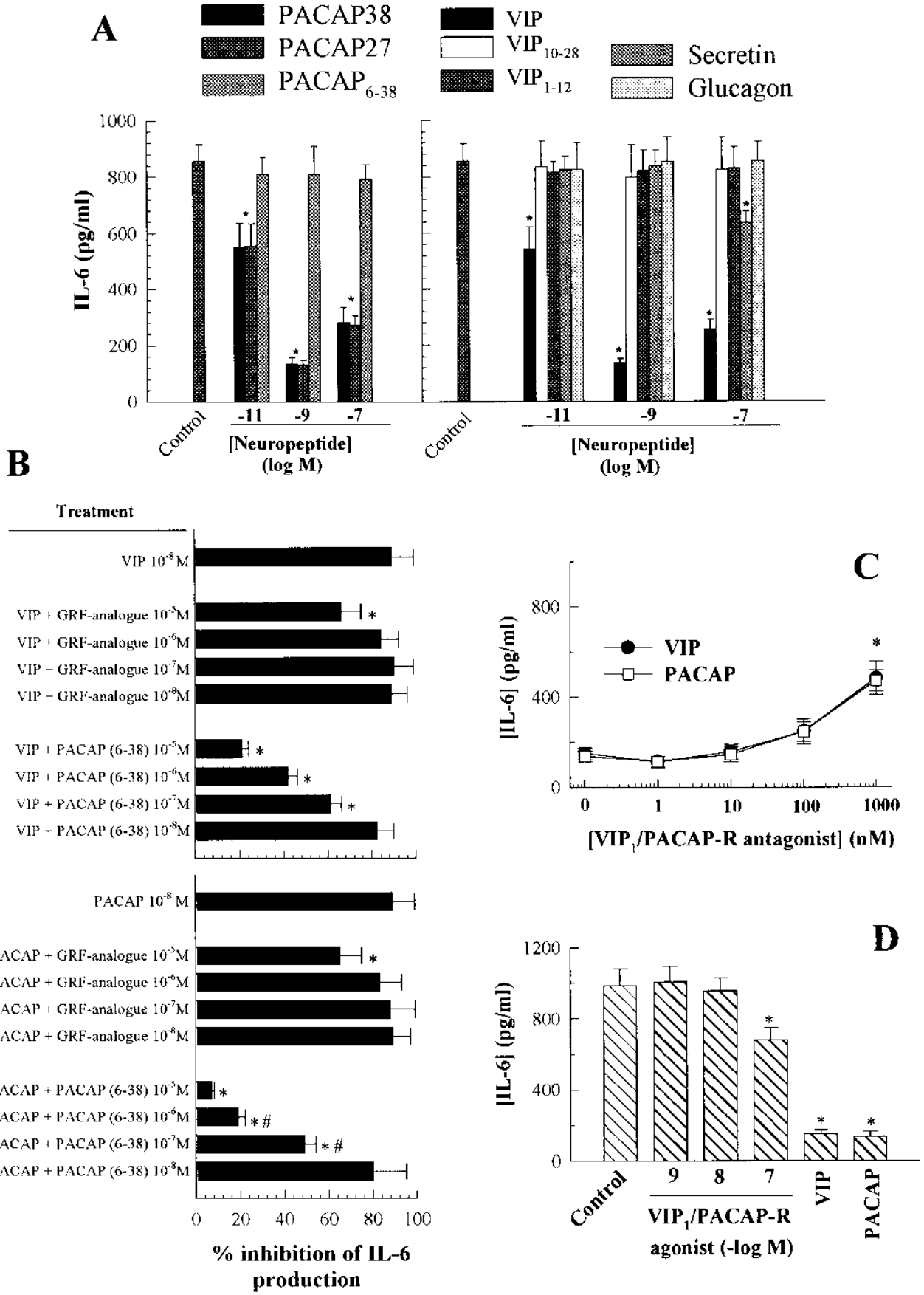
In addition, to determine whether contaminating lymphocytes might have contributed to the observed cytokine production, freshly isolated peritoneal suspensions were compared to monolayer cell preparations. Similar IL-6 values were obtained in supernatants harvested at 6 h from freshly isolated peritoneal exudate cultures (30–40% macrophages, 50–60% lymphocytes), and from monolayer cell preparations (approximately 96% macrophages), suggesting that the role of lymphocytes in this system is marginal (data not shown).

The inhibition of LPS-stimulated IL-6 production by VIP and PACAP is specific

The role of specific VIP/PACAP receptors in the inhibition of IL-6 production was investigated next. Previous reports showed that VIP and both PACAPs, PACAP38 and PACAP27, bind with similar affinities to VIP/PACAP receptors on different immune cells, including peritoneal macrophages [17–19]. In contrast, the structurally related peptides secretin and glucagon bind to the VIP/PACAP receptors poorly or not at all [20]. Also, it has been established that the VIP fragments 1–12 and 10–28, and the PACAP fragment 6–38 do not act as agonists in the inhibition of IL-2 and IL-10 [11, 12] and stimulation of IL-6 [unpublished results]. We first compared the effects of VIP, PACAP27, PACAP38, secretin, and glucagon, and of the VIP-and PACAP-fragments on IL-6 production. VIP, PACAP27, and PACAP38 inhibited to similar levels at all concentrations assayed (10^{-7}–10^{-11} M; **Fig. 2A**); secretin inhibited only at 10^{-7} M, and with a lower efficiency compared with VIP and PACAP. In contrast, glucagon, $PACAP_{6–38}$, and the two VIP fragments failed to inhibit IL-6 production (Fig. 2A). These results confirm the specificity of the VIP and PACAP and suggest that intact VIP and PACAP molecules are required for their inhibitory activity.

The classical VIP/PACAP receptors belong to three different types, i.e., the PACAP-R, which binds PACAP with much higher affinity than VIP, and VIP_1/PACAP-R and VIP_2/

Fig. 2. The inhibition of LPS-induced macrophage IL-6 production by VIP and PACAP is specific. (A) Comparative effects of VIP, PACAP38, VIP-related peptides, and VIP and PACAP fragments on IL-6 production by LPS-stimulated macrophages. Cells (5×10^5 cells/mL) were stimulated with LPS (10 ng/mL) in the presence or absence of three different concentrations of VIP, PACAP38, secretin, glucagon, $VIP_{1–12}$, $VIP_{10–28}$, PACAP27, $PACAP_{6–38}$ (10^{-7}, 10^{-9}, and 10^{-11} M). Supernatants were collected 6 h later and were assayed for IL-6 production by ELISA as described in Materials and Methods. Results are the mean ± SD of three experiments performed in duplicate. $*P < 0.001$ compared with controls containing stimuli in the absence of peptides. (B and C) Effect of PACAP-R- and VIP_1/PACAP-R-antagonists on the inhibitory activity of VIP and PACAP. Peritoneal macrophages (5×10^5 cells/mL) were stimulated with LPS (10 ng/mL) and treated simultaneously with VIP or PACAP (10^{-8} M) and different concentrations of two VIP_1/PACAP-R-antagonists, GRF-analog (B), and [Ac-His^1,D-Phe^2,K^{15},R^{16},L^{27}]VIP(3–7)-GRF(8–27) (C), or a PACAP-R-antagonist, PACAP 6–38 (B). Supernatants were collected 6 h later and assayed for IL-6 production. The percentage of inhibition was calculated by comparison with controls containing stimuli in the absence of peptides. IL-6 concentration in LPS-activated control samples was 875± 93 pg/mL. IL-6 production by unstimulated and LPS-stimulated macrophages was, respectively: 86 ± 16 and 872 ± 82 pg/mL for 10^{-5} M GRF analog; 91 ± 11 and 914 ± 102 pg/mL for 10^{-6} M VIP_1/PACAP-antagonist; and 81 ± 12 and 872 ± 89 pg/mL for 10^{-5} M $PACAP_{6–38}$. Each result is the mean ± SD of three to six experiments performed in duplicate. $*P < 0.001$ compared with samples treated with neuropeptides and without antagonists. $^{\#}P < 0.05$ with respect to corresponding VIP-treated samples. (D) Effect of a VIP_1/PACAP-R agonist on the inhibitory activity of VIP and PACAP. Macrophages (5×10^5 cells/mL) were stimulated with LPS (10 ng/mL), and treated with different concentrations of a newly synthesized VIP_1/PACAP-R agonist, [K^{15},R^{16},L^{27}]VIP(1–7)-GRF(8–27) VIP_1/PACAP-R agonist. Supernatants were collected 6 h later and assayed for IL-6 production. Results are the mean ± SD of four experiments performed in duplicate. $*P < 0.001$ compared with controls (LPS alone).


A
PACAP38
PACAP27
PACAP6-38
VIP
VIP10-28
VIP1-12
Secretin
Glucagon
IL-6 (pg/ml)
1000
800
600
400
200
0
Control
-11
-9
-7
[Neuropeptide] (log M)
B
Treatment
VIP 10-8 M
VIP + GRF-analogue 10-5M
VIP + GRF-analogue 10-6M
VIP + GRF-analogue 10-7M
VIP + GRF-analogue 10-8M
VIP + PACAP (6-38) 10-5M
VIP + PACAP (6-38) 10-6M
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VIP + PACAP (6-38) 10-8M
PACAP 10-8 M
PACAP + GRF-analogue 10-5M
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PACAP + PACAP (6-38) 10-5M
PACAP + PACAP (6-38) 10-6M
PACAP + PACAP (6-38) 10-7M
PACAP + PACAP (6-38) 10-8M
0 20 40 60 80 100
% inhibition of IL-6 production
C
[IL-6] (pg/ml)
800
400
0
VIP
PACAP
0 1 10 100 1000
[VIP1/PACAP-R antagonist] (nM)
D
[IL-6] (pg/ml)
1200
800
400
0
Control
9 8 7
VIP1/PACAP-R agonist (-log M)
VIP
PACAP

PACAP-R, which express similar affinities for VIP and PACAP. To elucidate the type of receptor involved in the inhibitory effect of VIP and PACAP on IL-6 production, we investigated the ability of $PACAP_{6–38}$, a PACAP antagonist with specificity for PACAP-R and VIP_2/PACAP-R [21], and of the (Ac-Tyr[1], D-Phe[2]) GRF (1–29) amide, a VIP_1/PACAP-R antagonist [22], to reverse the VIP and PACAP effects on IL-6 production. Macrophage monolayers were incubated with increasing concentrations of the antagonists (10^{-5} to 10^{-8} M) in the presence of 10^{-8} M VIP or PACAP. As shown in Figure 2B, the inhibitory effects of VIP and PACAP on IL-6 production were reversed by the PACAP-R-antagonist in a dose-dependent manner. The most effective antagonist concentration is 10^{-5} M, which almost completely abolishes the inhibitory effect of VIP and PACAP. In contrast, the VIP_1/PACAP-R antagonist (GRF analog) only partially reversed the inhibitory effect of VIP and PACAP and only at the highest concentration used (10^{-5} M). A second, recently developed, VIP_1/PACAP-R antagonist [Ac-His[1], D-Phe[2],K^{15},R^{16},L^{27}]VIP(3–7)-GRF(8–27) had a similar effect (Fig. 2C). In addition, a newly described VIP_1/PACAP-R agonist [K^{15},R^{16},L^{27}]VIP(1–7)-GRF(8–27) had a minimal inhibitory effect on IL-6 (Fig. 2D). The lack of effect of the VIP_1/PACAP-R antagonists and agonist suggest that this receptor type is minimally involved in the inhibition of LPS-induced IL-6 production by VIP and PACAP.

In contrast to VIP_1/ and VIP_2/PACAP-R, which are coupled primarily to the adenylate cyclase system, the PACAP-R is coupled to both the adenylate cyclase and inositol phosphate cascades [6]. To obtain further information regarding the type of VIP/PACAP receptor involved, we investigated the effect of H89 (a PKA inhibitor) and of calphostin C (a PKC inhibitor) on the inhibitory activity of VIP and PACAP. Calphostin C reversed the inhibitory effect of both VIP and PACAP in a dose-dependent manner (**Fig. 3A**). In contrast, H-89 had a very slight effect, and only at the highest concentration tested (Fig. 3B). These results indicate that PKC plays an important role in the inhibition of IL-6 by VIP and PACAP, whereas the adenylate cyclase pathway appears much less important. None of the antagonists and agonists affected significantly the cell number, viability, or IL-6 levels for nonstimulated macrophages cultured with or without VIP/PACAP (data not shown). None of the inhibitors affected the IL-6 levels in LPS-stimulated macrophages (data not shown). These results suggest that VIP and PACAP exert their inhibitory activity on IL-6 production through specific receptors that express similar affinities for VIP and PACAP, and are coupled to PKC.

Time course for the inhibitory effect of VIP and PACAP

To study the time course for the inhibitory activity of VIP and PACAP, we stimulated peritoneal macrophages with LPS (10 ng/mL), and added 10^{-8} M VIP or PACAP at different times before (1 and 2 h) and after (from 0 to 4 h) the initiation of the cultures. Supernatants were collected 6 h after the initiation of the cultures and were assayed for IL-6 production. The addition of VIP and PACAP up to 1 h after LPS stimulation resulted in significant levels of inhibition (72–84%; **Fig. 4A**). Later additions resulted in progressively lower degrees of inhibition

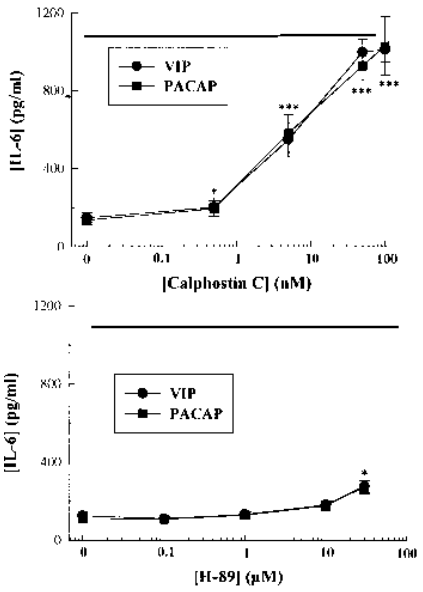

Fig. 3. Comparative effects of calphostin C (PKC inhibitor) and H-89 (PKA inhibitor) on LPS-induced IL-6 production in murine macrophages incubated with VIP or PACAP. Peritoneal macrophages (5×10^5 cells/mL) were stimulated with LPS (10 ng/mL), LPS plus VIP (10^{-8} M), or LPS plus PACAP (10^{-8} M) in the presence or absence of different concentrations of calphostin C or H-89. Supernatants were collected 6 h later and assayed for IL-6 production by ELISA. The solid horizontal lines represent control values from cultures incubated with LPS alone (984 ± 97 pg IL-6/mL). IL-6 production by macrophages stimulated with LPS (10 ng/mL) for 0.5, 5, 50, and 100 nM calphostin C was, respectively: 1022 ± 53, 1085 ± 131, 1051 ± 104, 1045 ± 112 pg IL-6/mL. IL-6 production by unstimulated macrophages was, respectively: 70 ± 7, 61 ± 7, 65 ± 5, and 63 ± 9 pg/mL for 0.5, 5, 50, and 100 nM calphostin C; and 96 ± 10, 68 ± 7, 68 ± 3, and 74 ± 5 pg/mL for 0.1, 1, 10, and 30 μM H-89. Each result is the mean ± SD of three to six experiments performed in duplicate. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ with respect to samples without protein kinese inhibitors.

(Fig. 4A). The addition of VIP and PACAP before LPS stimulation were ineffective in reducing IL-6 release. On the contrary, both neuropeptides added 1 or 2 h before LPS significantly stimulated the release of IL-6 (about 175 and 204% of stimulation for 1 and 2 h, respectively, in comparison with LPS-treated samples; Fig. 4A). To determine the minimum time required for the inhibitory effect of VIP and PACAP, peritoneal macrophages were cultured with LPS in the presence or absence of 10^{-8} M VIP or PACAP. Neuropeptides were removed by extensive washing at different times (from 5 min to 120 min), and cells were cultured in medium containing LPS without neuropeptides. A 15-min incubation with VIP or PACAP was enough to achieve maximum inhibition, although incubations of 5 min still result in significant inhibitory effects (Fig. 4B).

Effects of VIP and PACAP on IL-1β- and TNF-α-induced IL-6 release

To investigate whether the observed inhibition by VIP and PACAP was specific for endotoxin-induced cytokine release, or whether these neuropeptides might also act as inhibitors of IL-6 release by cytokine-activated macrophages, we determined the effects of VIP and PACAP on IL-1β- and TNF-α-induced IL-6 production. Both neuropeptides were ineffective in reducing

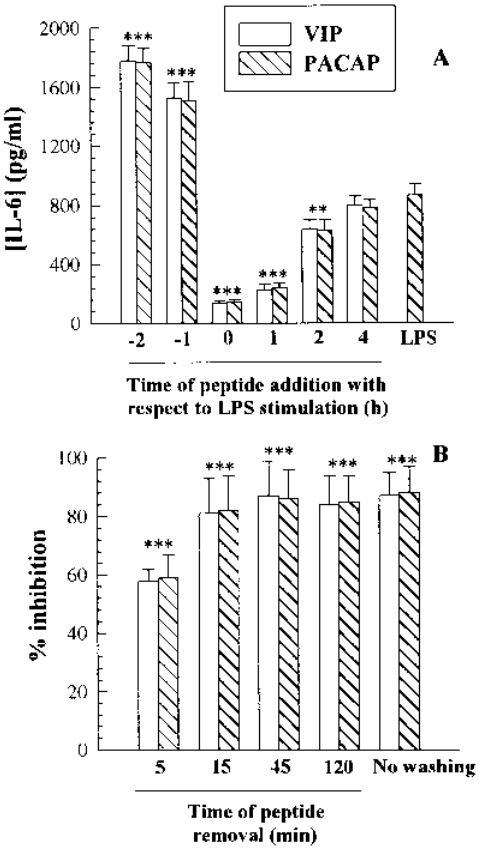

Fig. 4. Time course for the VIP and PACAP inhibitory activity on LPS-activated IL-6 production. (A) Peritoneal macrophages (5×10^5 cells/mL) were stimulated with LPS (10 ng/mL) at time 0. VIP or PACAP (10^{-8} M) were added at different hours before or after the addition of LPS. Supernatants were collected 6 h after the addition of LPS and assayed for IL-6 production as described in Materials and Methods. (B) Peritoneal macrophages (5×10^5 cells/mL) were stimulated with LPS (10 ng/mL) in the presence or absence of VIP or PACAP (10^{-8} M). Both neuropeptides were removed at different times by washing (three times with serum-free medium) and the cells were then resuspended in medium containing only LPS (10 ng/mL) and cultured for an additional 6 h. Control cultures containing LPS and no neuropeptides were washed and recultured under the same conditions. Supernatants were collected and the IL-6 content was determined as described in Materials and Methods. Percentage inhibition was calculated by comparing each experimental culture with its control. Each result is the mean ± SD of three experiments performed in duplicate. $^{**}P < 0.01$ and $^{***}P < 0.001$ with respect to LPS-stimulated controls without neuropeptides.

IL-6 production after stimulation with IL-1β or TNF-α for 6 h. On the contrary, VIP and PACAP significantly stimulated the release of IL-6 in a dose-dependent manner (**Fig. 5A**), showing a synergistic effect with these cytokines. The stimulatory effect of VIP/PACAP on cytokine-induced IL-6 production extends over longer periods of time, with a maximal effect at 18 h (Fig. 5B).

VIP and PACAP regulate IL-6 production at a transcriptional level

Having demonstrated that VIP and PACAP had an inhibitory effect on endotoxin-induced IL-6 production, we determined whether this action occurs at a transcriptional level. We stimulated peritoneal macrophages with LPS in the presence or absence of 10^{-8} M VIP or PACAP. Total RNA was prepared from 3 h cultures and subjected to Northern blot analysis. Although no IL-6 mRNA was detectable in unstimulated cells, a high level of IL-6 mRNA was present in LPS-stimulated macrophages, and the hybridization signal was strongly reduced in the presence of either VIP or PACAP (**Fig. 6**). These results indicate that VIP and PACAP inhibit IL-6 production at the transcriptional level in LPS-stimulated macrophage cultures.

VIP and PACAP inhibit LPS-induced IL-6 production in vivo

Finally, an attempt was made to reproduce the in vitro observations in vivo. To this end, mice were treated with VIP or PACAP or with vehicle control solution, followed by the administration of LPS (from *S. enteridis*, 25 μg/mouse). The four experiments depicted in **Table 2** show that both neuropeptides (a single dose of 5 nmol/mouse) decrease the concentration of IL-6 induced by LPS in serum and extracellular fluid (peritoneal lavage). Other authors have previously shown that circulating IL-6 peaks 3 h after the inoculation of LPS. We used the same experimental protocol to analyze IL-6 mRNA in fresh peritoneal cells by Northern blot analysis. As seen in **Figure 7**, VIP and PACAP significantly reduce the expression of IL-6 mRNA at 1.5 h post-endotoxin.

DISCUSSION

Although VIP affects a variety of immune functions, its primary immunomodulatory function seems to be anti-inflammatory in nature. In this respect, VIP and PACAP has been shown to attenuate reperfusion injury after ischemia of brain [23] and lung [24]. VIP has a protective effect on the acute lung inflammation caused by HCl [25], xanthine/xanthine oxide [26], and toxic oxygen metabolites [27]. In this study, we investigated the effects of VIP and PACAP on the production of IL-6, a pro-inflammatory cytokine, by endotoxin-activated macrophages. Both neuropeptides exhibit a dual effect on the IL-6 production by peritoneal macrophages. Whereas VIP and PACAP enhance IL-6 secretion in unstimulated macrophages and in macrophages stimulated with very low LPS concentrations (1–10 pg/mL), both neuropeptides inhibit, with similar dose-response curves, the release of IL-6 from macrophages stimulated with LPS in the dose range from 100 pg/mL to 10 μg/mL. These results suggest that VIP/PACAP play different physiological roles depending on the dose of the inflammatory stimulus. These effects were found to be dose-dependent within a wide range of VIP/PACAP concentrations, with maximum effects at 10^{-9}–10^{-8} M. This is a dose range in which VIP and PACAP has been shown to produce immunological and biological effects in other systems [1, 2, 12, 18]. The lower inhibitory effect observed at high neuropeptide concentrations might be due to a desensitization process through the internalization of neuropeptide receptors [28].

The time course indicates that, similar to IL-10 [12], the effect of VIP and PACAP on IL-6 production occurs rapidly because 5- to 15-min incubations with either neuropeptide are sufficient to achieve a maximum effect. In addition, the

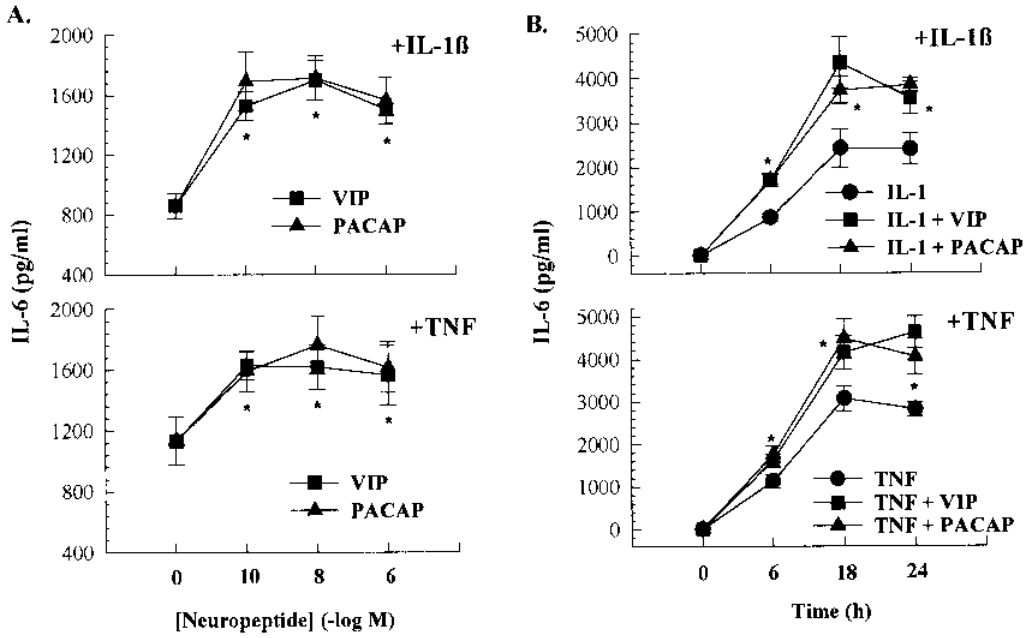

Fig. 5. Modulation of IL-1β-and TNF-α-induced IL-6 release by VIP and PACAP. (A) Macrophages (5×10^5 cells/mL) were incubated with mrIL-1β (100 U/mL) or mrTNF-α (10 ng/mL) in the absence or presence of different concentrations of VIP or PACAP38. The supernatants were collected 6 h later, and the IL-6 release was determined by ELISA. (B) Macrophages (5×10^5 cells/mL) were incubated with mrIL-1β (100 U/mL) or mrTNF-α (10 ng/mL) in the absence or presence of VIP or PACAP (10^{-8} M) for 24 h. At the indicated time points, supernatants were collected and the IL-6 release was determined by ELISA. Results are shown as mean ± SD of three experiments performed in duplicate. Asterisks indicate a statistically significant difference compared with controls containing stimuli in the absence of neuropeptides ($P < 0.001$).

inhibitor has a refractory period of 2–3 h, after which the inhibitory activity of VIP or PACAP is reduced significantly.

The specificity of the VIP and PACAP action on peritoneal macrophages is supported by the comparative effect of VIP, PACAP38, PACAP27, secretin, and glucagon, all structurally related peptides. VIP and both PACAP forms bind with high affinity, secretin with a much lower affinity, and glucagon does not bind to the VIP/PACAP receptors [6, 7]. The effects of the different peptides on the LPS-stimulated IL-6 production follows the same potency order. Furthermore, the lack of effects of VIP- and PACAP fragments ($VIP_{10–28}$, $VIP_{1–12}$, and $PACAP_{6–38}$) on endotoxin-induced IL-6 release, indicates that intact VIP and PACAP molecules are required. Similar results were previously obtained for IL-2 and IL-10 inhibition in spleen cells and thymocytes [11, 12].

The nature of the macrophage VIP/PACAP receptors that mediate the inhibtion of IL-6 production is not clear. We reported recently that both PACAP-R and VIP_1/PACAP-R

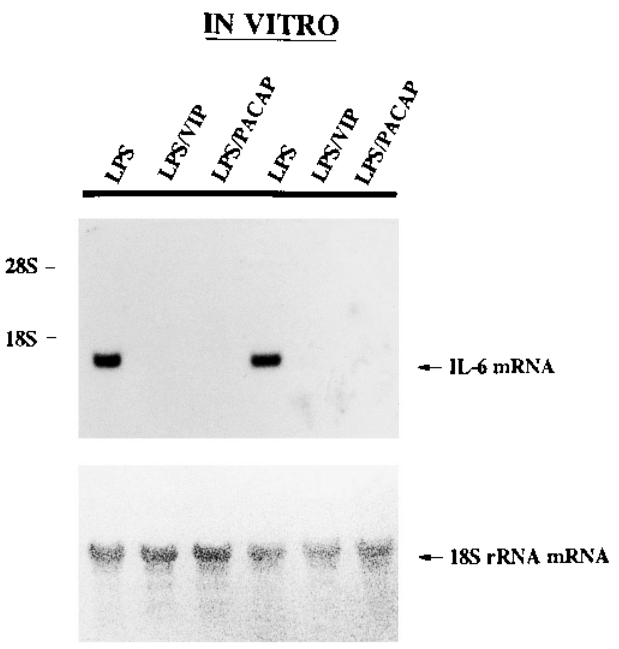

Fig. 6. Inhibitory effects of VIP and PACAP on LPS-induced IL-6 mRNA expression. Macrophages (10^7 cells) were stimulated with LPS (10 ng/mL) and incubated without or with VIP or PACAP (10^{-8} M) for 3 h. Total RNA was extracted and blotted onto nylon membranes. Expression of IL-6 and 18S rRNA mRNA in peritoneal macrophages was analyzed by Northern blot analysis. One representative experiment of three is shown.

TABLE 2. In Vivo Effects of VIP and PACAP on LPS-Stimulated IL-6 Production

Treatment	Time (h)	IL-6 levels (pg/mL) Serum	Peritoneal exudate
LPS (controls)	2	1504 ± 124	4546 ± 421
LPS + VIP	2	626 ± 44*	2362 ± 325*
LPS + PACAP	2	386 ± 25*	2540 ± 286*
LPS (controls)	3	1727 ± 158	5092 ± 215
LPS + VIP	3	973 ± 132*	3356 ± 286*
LPS + PACAP	3	756 ± 60*	2583 ± 83*

Mice were injected intraperitoneally with LPS (25 μg/mouse), LPS plus VIP (5 nmol/mouse), or LPS plus PACAP (5 nmol/mouse). IL-6 levels were measured 2 or 3 h later as described in Materials and Methods. Results are expressed as mean pg/mL ± SD from four experiments performed in duplicate. * $P < 0.001$ vs control group.

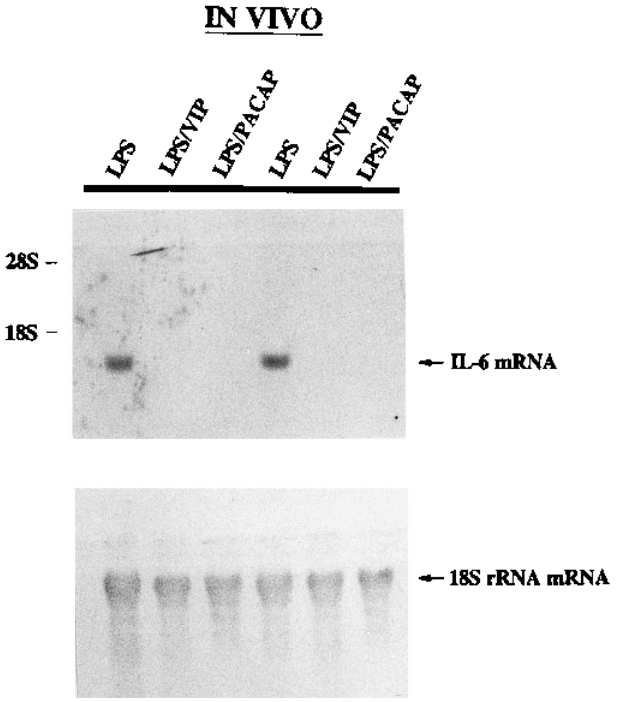

Fig. 7. In vivo effects of VIP and PACAP on IL-6 mRNA in endotoxemic mice. Mice were injected intraperitoneally with LPS (25 μg/mouse, lane 1 and 4), LPS plus VIP (5 nmol/mouse, lane 2 and 5), or LPS plus PACAP (5 nmol/mouse, lane 3 and 6) as described in Materials and Methods. Expression of IL-6 and 18S rRNA mRNA in the peritoneal exudate was analyzed by Northern blot analysis 1.5 h post-LPS. One representative experiment of two is shown.

mRNA are expressed in rat macrophages [19, 29], and both high-affinity and low-affinity VIP/PACAP binding sites were identified on peritoneal macrophages [19, 30]. The K_d of the high-affinity binding sites for VIP and PACAP38 (0.6–1.44 nM) corresponds to the neuropeptide concentrations (10^{-9}–10^{-8} M) that showed the highest modulatory effect in our study. The lack of effect of both VIP_1/PACAP-R antagonists and agonist eliminates this receptor as a possible mediator. $PACAP_{6-38}$, an antagonist with specificity for both the PACAP-R and VIP_2/PACAP-R [21] is fully active in reversing the inhibitory effect of both VIP and PACAP. Therefore, either receptor is a viable candidate. However, the VIP_2/PACAP-R is coupled primarily to the adenylate cyclase system, and H89 (a specific PKA inhibitor) [31] does not affect the inhibitory effect of either VIP or PACAP. The PACAP-R, which is coupled to both the adenylate cyclase and PLC systems, appears to be a much better candidate because calphostin C (a specific and potent PKC inhibitor) [33] does indeed reverse the inhibitory effect of VIP/PACAP. However, a major characteristic of the PACAP-R is its preference for PACAP over VIP, and in our system both VIP and PACAP express similar potencies for the inhibition of IL-6. Previous reports from our laboratory indicated that VIP/PACAP also affect macrophage phagocytosis, adherence, and mobility with similar efficiencies, and that these effects are mediated, at least partially, through PKC [33–36]. Although the PACAP-R expresses higher affinity for PACAP than for VIP, the difference in affinities varies significantly with the cellular origin of the receptor. Reported differences range from three orders of magnitude in a neuroblastoma cell line [37] to 10-fold in an osteoblast-like cell line [38]. Our previous studies indicate that the high-affinity VIP/PACAP binding sites in peritoneal macrophages express a slight preference for PACAP27 and -38 over VIP (K_d 0.64 nM and 0.66 nM for PACAP27 and -38, and 1.44 nM for VIP) [19]. All these results point to the existence in peritoneal macrophages of a subtype of PACAP-Rs coupled to the PLC/PKC pathway and expressing only a slight preference for PACAP, which mediate several of the biological effects of VIP/PACAP such as inhibition of IL-6 and stimulation of macrophage phagocytosis, mobility, and adherence.

In contrast to the broad inhibitory effects of VIP and PACAP on LPS-induced IL-6 release, both neuropeptides were ineffective in reducing release of IL-6 by macrophages stimulated with either IL-1β or TNF-α. On the contrary, we found that VIP and PACAP significantly increased the IL-6 levels in the culture supernatants after 6 h of stimulation with both inflammatory cytokines. This potentiating effect was evident during a 24-h incubation period. These findings show that VIP and PACAP have opposing effects on the release of IL-6 by activated macrophages, depending on the nature of the stimulus. Similar results have been described in human monocytes for another anti-inflammatory substance, the endogenous nucleoside adenosine [39].

Our previous experiments regarding VIP modulation of cytokine expression indicated that the inhibitory effect of VIP on IL-2/IL-10 and IL-4 production results from different molecular mechanisms, i.e., transcriptional vs. posttranscriptional regulation, respectively [10]. The present study indicates that, similar to IL-2 and IL-10, and in contrast to IL-4, the inhibitory effect of VIP and PACAP occurs through a transcriptional mechanism. Northern analysis indicates a significant decrease in the IL-6 mRNA by macrophages stimulated with LPS on treatment with VIP or PACAP38. It remains to be established whether the diminution in steady state IL-6 mRNA levels results from a decrease in de novo transcriptional rate, message destabilization, or both.

Several lines of evidence suggest that the production and expression of VIP and of VIP binding sites are regulated by inflammatory mediators. IL-6 responsive elements are present in the promoter of the VIP gene in a neuroblastoma cell line, and a carboxyl-terminal sequence of the signal-transducing receptor subunit gp130 is required for the full activation of this IL-6 responsive element [40], suggesting that IL-6 is involved in the production of VIP. Kaltreider et al. [41] have recently found an up-regulation of VIP and VIP receptors in a murine model of immune inflammation in lung. Similarly, VIP binding sites on human mononuclear leukocytes are up-regulated during prolonged strain and energy deficiency after treatment with glucocorticoids, and in casein-elicited peritoneal macrophages [42]. These findings suggest that the production of mediators by inflammatory cells may stimulate VIP and VIP-receptor expression in lymphocytes/monocytes and that the VIP/VIP-R interactions may negatively regulate the local inflammatory response.

The in vitro inhibitory effect of VIP/PACAP on LPS-induced IL-6 production correlates with the in vivo inhibition on both IL-6 expression and release in endotoxemic mice. Intraperito-

neal LPS administration results in significant increases in serum IL-6 and in increased IL-6 mRNA and IL-6 release by peritoneal cells. The LPS-induced increase in IL-6 in vivo is partially prevented by simultaneous intraperitoneal administration of VIP or PACAP. VIP and PACAP have been described as components of the lymphoid microenvironment, including the peritoneal immune population. Indeed, we have previously reported that VIP is produced by peritoneal lymphocytes [29], and Gaytan et al. [5] found PACAP immunoreactivity in lymphocytes from different lymphoid organs. Therefore, the in vivo effect of VIP and PACAP on IL-6 production, the expression and production of both neuropeptides and their receptors in peritoneal immune cells, together with the concept of a VIP-and PACAP-mediated general immunoregulatory feedback mechanism, suggest that these neuropeptides might be endogenous modulators of inflammatory cytokine production by activated immune cells.

In conclusion, in this report we show that VIP and PACAP have differential effects on IL-6 production by peritoneal macrophages depending on the nature and dose of the inflammatory stimuli. Based on these results, we postulate that in the absence of a stimulus or after low doses of LPS, VIP and PACAP play a role in immune system homeostasis, whereas in toxicity conditions, induced by high doses of LPS, VIP and PACAP regulate the excessive release of IL-6 in order to reduce inflammation or shock. Because VIP degrades rapidly, stable synthetic VIP-analogs may have significant clinical application. Such analogs are presently being developed in several laboratories. Moreover, a better understanding of the mechanisms involved in interaction between VIP/PACAP and proinflammatory cytokines such as IL-6 may lead to new ways of blocking their pathological effects in inflammation or shock.

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