Vasoactive Intestinal Peptide and Pituitary Adenylate Cyclase-Activating Polypeptide Inhibit Endotoxin-Induced TNF-α Production by Macrophages: In Vitro and In Vivo Studies[1]

Mario Delgado,[2]*‡ David Pozo,† Carmen Martinez,* Javier Leceta,* Juan Ramon Calvo,† Doina Ganea,‡ and Rosa P. Gomariz*

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. The production and release of cytokines by activated phagocytes are important events in the pathogenesis of ischemia-reperfusion injury. There is abundant evidence that the proinflammatory cytokine TNF-α is an important mediator of shock and organ failure complicating Gram-negative sepsis. VIP has been shown to attenuate the deleterious consequences of this pathologic phenomenon. In this study we have investigated the effects of VIP and the structurally related neuropeptide pituitary adenylate cyclase-activating polypeptide (PACAP38) on the production of TNF-α by endotoxin-activated murine peritoneal macrophages. Both neuropeptides rapidly and specifically inhibit the LPS-stimulated production of TNF-α, exerting their action through the binding to VPAC1 receptor and the subsequent activation of the adenylate cyclase system. VIP and PACAP regulate the production of TNF-α at a transcriptional level. In vitro results were correlated with an inhibition of both TNF-α expression and release in endotoxemic mice in vivo. The immunomodulatory role of VIP in vivo is supported by the up-regulation of VIP release in serum and peritoneal fluid by LPS and proinflammatory cytokines such as TNF-α, IL-1β, and IL-6. These findings support the idea that under toxicity conditions associated with high LPS doses, VIP and PACAP could act as protective mediators that regulate the excessive release of TNF-α to reduce inflammation or shock. *The Journal of Immunology,* **1999, 162: 2358–2367.**

Current evidence indicates that macrophages play an important role in the pathogenesis of the inflammatory responses by their ability to produce cytokines such IL-1β, IL-6, and TNF-α. TNF-α is a monocyte-macrophage-derived cytokine that acts as an important mediator in the defense mechanism of the host in response to bacterial colonization or invasion and causes immunopathologic disorders when secreted in excess (1). The toxicity caused by Gram-negative bacteria has been ascribed to LPS, an outer membrane component of bacteria. LPS represents one of the most potent inducers of TNF-α and in high concentrations causes tissue injury, fever, disseminated vascular coagulation, and septic shock, often resulting in death (1).

Vasoactive intestinal peptide (VIP)[3] and pituitary adenylate cyclase-activating polypeptide (PACAP) are, respectively, 28- and 38-amino acid peptides that have 65% homology and elicit a broad spectrum of biological functions, including actions on natural and acquired immunity (2). Both VIP and PACAP interact with a family of three VIP/PACAP receptors: VIP_1 receptor (VPAC1), which is the classical receptor, shows similar affinities for VIP and PACAP and stimulates almost exclusively the adenylate cyclase system. VPAC2 has similar affinities for both peptides, activates the adenylate cyclase system, and seems to be coupled to a calcium chloride channel in transfected cells. The third type of receptor is the PACAP-preferring receptor (PAC1) that recognizes PACAP much more potently than VIP and activates both adenylate cyclase and phospholipase C. Macrophages have been described to possess VPAC1 (3, 4), and we have recently reported PAC1 receptor gene expression in rat peritoneal macrophages (5).

Actions on natural and acquired immunity of VIP and PACAP seem to be mediated through effects on the production and release of cytokines. Thus, VIP stimulates IL-5 release from schistosome granulomas and splenic T lymphocytes, and inhibits the expression and secretion of IL-2, IL-4, and IL-10 in stimulated peripheral T cell and thymocytes cultures (6). Moreover, we have recently studied the effects of VIP and PACAP on the production of the inflammatory cytokine IL-6 (7). We showed that both neuropeptides exhibited a dual effect on IL-6 production by peritoneal macrophages. Whereas VIP and PACAP inhibited the release of IL-6 from macrophages stimulated with high LPS doses, the two neuropeptides enhanced IL-6 secretion in macrophages unstimulated and stimulated by low LPS doses. To clarify the roles played by VIP and PACAP in vivo and in vitro in inflammation, we addressed the following objectives in this study: 1) to determine the in vitro and in vivo effects of both peptides on LPS-induced TNF-α production and release by macrophages, 2) to investigate the specific receptor as well as the intracellular signal pathways involved,

*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, NJ 07102

Received for publication July 6, 1998. Accepted for publication November 9, 1998.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by Grants PB94-0310 (to R.P.G.) and PB94-1434 (to J.R.C.) from the Spanish Department of Education and Science, Grant PHS AI41786 (to D.G.), and a postdoctoral fellowship from the Spanish Department of Education and Science (PM98-0081 to M.D.).

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

[3] Abbreviations used in this paper: VIP, vasoactive intestinal peptide; PACAP, pituitary adenylate cyclase-activating polypeptide; GRF, growth hormone-releasing factor; mr, murine recombinant; PKC, protein kinase C; PKA, protein kinase A; ABTS, 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid.

 0022-1767/99/$02.00

and 3) to elucidate the possible existence of a negative feedback mechanism in the regulation of proinflammatory cytokine production by endogenous VIP/PACAP.

Materials and Methods

Animals

Female BALB/c mice, 6–10 wk of age (weighing 19–20 g), were purchased from Iffa Credo (France). Mice were kept in the animal house for at least 1 wk before use in a temperature-controlled room with a 12-h light, 12-h dark cycle and were allowed free access to standard laboratory chow and water.

Abs and reagents

Synthetic VIP, PACAP38, PACAP27, VIP_{1-12}, and VIP_{10-28} were purchased from Novabiochem (Laufelfingen, Switzerland). The VPAC1 antagonist [Ac-His^1,D-Phe^2,K^{15},R^{16},L^{27}]VIP_{3-7}-GRF_{8-27} and the VPAC1 agonist [K^{15},R^{16},L^{27}]VIP_{1-7}-GRF_{8-27} were donated by Dr. Robberecht (Université Libre de Bruxelles, Brussels, Belgium). The VIP antagonist [*N*-Ac-Tyr^1,D-Phe^2]-GRF_{1-29} amide was obtained from Cambridge Research BioChemicals (Wilmington, DE), and $PACAP_{6-38}$, secretin, glucagon, and biotinylated VIP were obtained from Peninsula Laboratories (Belmont, CA). Oligonucleotides were synthesized by Pharmacia Biotech (Uppsala, Sweden). The blocking reagent, digoxigenin oligonucleotide tailing kit, anti-digoxigenin-AP (Fab), and the chemiluminescent substrate for alkaline phosphatase (CSPD) were obtained from Boehringer Mannheim (Mannheim, Germany). Murine recombinant (mr) IL-1β, mrTNF-α, mrIL-6, and capture and biotinylated monoclonal anti-murine TNF-α Abs were purchased from PharMingen (San Diego, CA). LPS (from *Escherichia coli* 0111:B4, *E. coli* 0127:B8, and *S. enteridis*), FITC-0111:B4 LPS, calphostin C, 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS substrate), *N*-laurylsarcosyl, malic acid, forskolin, PMA, SDS, EDTA, EGTA, and avidin-peroxidase were purchased from Sigma (St. Louis, MO), and *N*-[2-(*p*-bromocinnamyl-amino)ethyl]-5-iso-quinolinesulfonamide (H-89) was obtained from ICN Pharmaceuticals (Costa Mesa, CA). Anti-VIP mAb was a generous gift from Dr. Wong (University of California School of Medicine, Los Angeles, CA).

Preparation of macrophages and cell cultures

Thioglycolate-elicited macrophages were prepared as reported previously (8). Briefly, 2 ml of 4% Brewer's thioglycolate medium (Difco, Detroit, MI) was injected i.p. into mice, and peritoneal exudate cells were collected on day 4 by flushing peritoneal cavity with ice-cold RPMI 1640 medium (Life Technologies, Grand Island, NY). 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) containing 0.01 M HEPES buffer, 1 mM pyruvate, 0.1 M nonessential amino acids, 2 mM L-glutamine, 50 μM 2-ME, 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/well in a final volume of 1 ml. The cells were incubated at 37°C for 2 h so they would 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 morphologic and phagocytic criteria and by flow cytometry using MAC-3 Ab (clone M3/84, CD107b). Macrophage monolayers were incubated in RPMI 1640 complete medium and stimulated with different concentrations of LPS (from 10 pg/ml to 10 μg/ml) in the presence or the absence of VIP or PACAP38 (from 10^{-13} to 10^{-7} M) at 37°C in a humidified incubator with 5% CO_2. In some experiments, macrophages were stimulated with IL-1β (100 U/ml). Cell-free supernatants were harvested at the designated time points and kept frozen (−20°C) until assayed for TNF-α production by ELISA. When indicated, to determine the effect of calphostin C, H-89, forskolin, PMA, and the VIP and PACAP antagonists, 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 TNF-α

The amount of TNF-α present in supernatants was determined using a murine TNF-α capture ELISA assay essentially as described previously (9). Briefly, a capture monoclonal anti-murine TNF-α Ab (clone MP6-XT22) was used to coat microtiter plates (Corning ELISA plates) at 2 μg/ml at 4°C for 16 h. After washing and blocking with PBS containing 3% BSA, culture supernatants were added to each well for 12 h at 4°C. Unbound material was washed off, and a biotinylated rabbit anti-mouse TNF-α mAb was added at 2 μg/ml for 2 h. Bound Ab 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 addition of the substrate. A standard curve was constructed using various dilutions of murine rTNF-α in PBS containing 10% FCS. The amount of TNF-α in the culture supernatants was determined by extrapolation of absorbances to the standard curve. The ELISA was specific for murine TNF-α (did not cross-react with human or rat TNF-α or with other murine cytokines, such as IL-10, IL-6, IL-2, IL-3, IL-4, IL-5, IL-1α, IL-1β, and IFN-γ). The sensitivity of the assay was 10 pg of TNF-α/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 the 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, Houston, TX) according to the manufacturer's instructions. Briefly, macrophage monolayer (2×10^7 cells) was lysed in Ultraspec RNA reagent, followed by chloroform extraction and isopropanol precipitation. The RNA was resuspended in diethylpyrocarbonate-treated water and quantitated spectrophotometrically at 260/280 nm. Twenty micrograms of total RNA from each sample was electrophoresed on 1.2% agarose-formaldehyde gels, transferred to Nytran membranes (Schleicher & Schuell, Keene, NJ), and cross-linked to the nylon membrane using UV light (Linus, Cultek SL, Spain).

The probe for TNF-α was an oligonucleotide (5′-TTGACCT CAGCGCTGAGTTGGTCCCCCTTCTCAGCTGGAAGACT-3′) designed from the murine TNF-α mRNA (10). The probe for the murine 18S ribosomal RNA, as a quantity control for RNA, was an oligonucleotide (5′-CCAATTA CAGGGCCTCGAAAGAGTCCTGTA-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 each time in diethylpyrocarbonate-water and prehybridized at 68°C for 3 h in prehybridization buffer containing 5× SSC, 1% blocking reagent (w/v), 0.1% *N*-laurylsarcosyl (w/v), and 0.02% SDS (w/v). Hybridization was performed at 60°C for 16 h in the same prehybridization buffer plus 25 ng/ml of TNF-α 3′-labeled oligonucleotide. Thereafter, the blots were washed twice for 5 min each time in 2× SSC/0.1% SDS (w/v) at room temperature, followed by washing twice for 5 min each time to a final stringency of 0.1× SSC/0.1% SDS (w/v) at 60°C. To detect the hybridization signal, the blots were blocked for 30−45 min in 0.1 malic acid (pH 7.5), 0.15 M NaCl, and 1% blocking reagent; further incubated with 1/5000 anti-digoxigenin Ab conjugated with alkaline phosphatase for 30 min in the same buffer; and developed by chemiluminescence in 0.1 M Tris-HCl (pH 9.5), 0.1 M NaCl, and 0.25 mM CSPD. The chemiluminescent signals were obtained using Kodak X-OMAT AR film (Sigma) at room temperature. The membranes were stripped twice for 30 min each time in 50 mM Tris-HCl (pH 8.0) containing 5% *N*,*N*-dimethyl-formamide (v/v) and 1% SDS (w/v) at 68°C and were rehybridized with the 18S ribosomal RNA-labeled antisense oligonucleotide at 55°C.

In vivo quantitation of TNF-α levels

Female mice (6–10 wk old) were randomized to receive a single i.p. dose of LPS (25 μg/mouse), LPS plus VIP (5 nmol/mouse), or LPS plus PACAP38 (5 nmol/mouse). After 2 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 TNF-α ELISA analysis. The peritoneal suspension was centrifuged for 5 min at $1800 \times g$, and cell-free supernatant was harvested and assayed for TNF-α ELISA. The peritoneal cells were subjected to Northern blot analyses as described above.

Determination of VIP levels

VIP production was determined using a competitive ELISA. Total peritoneal cell suspension (5×10^6 cells) was incubated with various stimuli as indicated in *Results*. After different times, the supernatants (1 ml) were collected and lyophilized. A capture anti-VIP mAb (clone CURE.V55) was used to coat microtiter plates (Corning ELISA plates) at 2.5 μg/ml at 4°C for 16 h. After washing and blocking with PBS containing 3% BSA, lyophilized culture supernatants resuspended in 100 μl PBS/10% FCS containing 0.5 ng/ml biotinylated-VIP were added to each well for 12 h at 4°C. Bound biotinylated VIP was detected by addition of avidin-peroxidase for 30 min followed by addition of the ABTS substrate solution. Absorbances at 405 nm were determined 30 min after addition of the substrate. A standard curve was constructed using various dilutions of VIP in PBS containing 10% FCS. The amount of VIP in the culture supernatants was determined by extrapolation of absorbances to the standard curve. The ELISA was specific for VIP (did not cross-react with secretin, glucagon,

PACAP27, PACAP38, VIP_{1-12}, or VIP_{10-28}). The sensitivity of the assay was 20 pg of VIP/ml.

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 Student's *t* test followed by Scheffe's F test, with $p < 0.05$ as the minimum significant level.

Results

VIP and PACAP inhibit LPS-induced TNF-α production

To determine the effects of VIP and PACAP on TNF-α production, macrophages were stimulated with LPS, and supernatants were harvested at different time points and assayed for TNF-α production by ELISA. Stimulation of macrophages with LPS for 6 h resulted in a dose-dependent release of TNF-α (Fig. 1*A*). The addition of VIP or PACAP to LPS-stimulated cultures resulted in an inhibition of TNF-α production by macrophages (Fig. 1, *A* and *B*). The dose-response curves were similar for VIP and PACAP (Fig. 1*B*), showing maximal effects at 10^{-8}–10^{-9} M. During stimulation with 1–10 ng/ml LPS, the inhibitory activity of VIP and PACAP was the most pronounced, with maximum values of 57%, showing an IC_{50} (i.e., the concentration of neuropeptide producing 50% of maximal inhibition) of 80 pM (Table I). The time curves indicate that the TNF-α release was significantly inhibited by VIP and PACAP as early as 3 h, with the maximum inhibitory effect after 6 h of culture (Fig. 1*C*). In addition, 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 these neuropeptides during a 24-h incubation period. The reduction of TNF-α release was maintained throughout the 24-h incubation period, indicating that VIP and PACAP do not delay but, rather, attenuate TNF-α release (Fig. 1*C*). Neither VIP nor PACAP merely alters the time course of TNF-α production by macrophages stimulated with LPS.

Since the highest inhibition of TNF-α 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, as neither VIP nor PACAP affected cell numbers or viability for stimulated macrophages after 8 h of culture (viabilities ranged from 87–96% with or without peptides).

In addition, to determine whether contaminating lymphocytes might have contributed to the observed cytokine production, freshly isolated peritoneal suspensions were stimulated with LPS in the presence or the absence of VIP or PACAP. Similar TNF-α values were obtained in supernatants collected at 6 h from freshly isolated peritoneal exudate cultures (50–60% macrophages, 30–40% lymphocytes) and from monolayer cell preparations containing about 96% macrophages, suggesting that the role of lymphocytes in this system is marginal (data not shown).

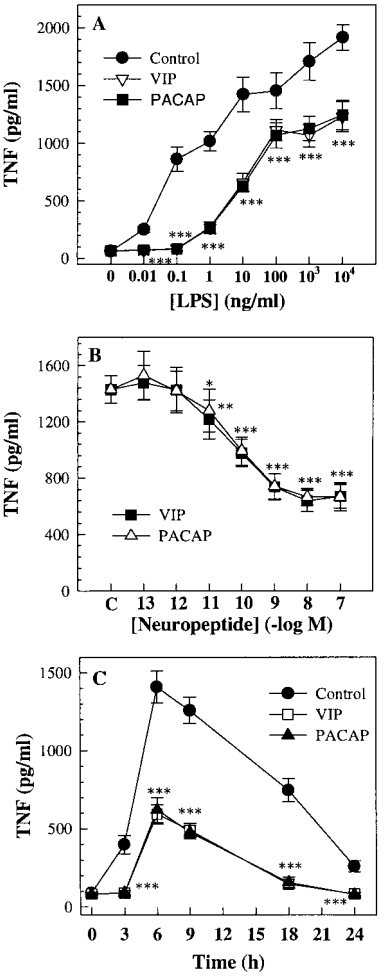

FIGURE 1. VIP and PACAP inhibit TNF-α production by LPS-stimulated murine macrophages. *A*, Peritoneal macrophages (5×10^5 cells/ml) were stimulated with a range of LPS concentrations (10 pg/ml to 10 μg/ml) in the absence or the presence of 10^{-8} M VIP or PACAP38. After a 6-h incubation period, the supernatants were collected, and TNF-α release was determined by ELISA. *B*, Dose-response curve for the inhibitory effect of VIP and PACAP on LPS-stimulated TNF-α production. Macrophages (5×10^5 cells/ml) were incubated with LPS (10 ng/ml) and a range of concentrations of either VIP or PACAP for 6 h. Control cultures (C) were incubated with LPS alone. The supernatants were collected, and TNF-α release was determined by ELISA. *C*, Time course for the inhibitory effect of LPS-stimulated TNF-α production by VIP and PACAP. Peritoneal macrophages (5×10^5 cells/ml) were stimulated with LPS (10 ng/ml) in the absence or the presence of 10^{-8} M VIP or PACAP; supernatants collected at different times were assayed for TNF-α production by ELISA. Each result is the mean ± SD of 10 separate experiments performed in duplicate. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (vs control cultures with LPS alone).

Inhibition of LPS-stimulated TNF-α production by VIP and PACAP is specific

After having established that VIP and PACAP inhibited TNF-α production by LPS-stimulated macrophages, we investigated whether these effects could be related to the occupancy of specific receptors. Previous reports showed that VIP and both PACAPs, PACAP38 and PACAP27, bind with similar affinities to VIP/PACAP receptors on different immune cells, including macrophages (5, 11, 12). In contrast, the structurally related peptides secretin and glucagon bind to the VIP/PACAP receptors poorly or not at all. Also, it has been established that VIP fragments 1–12 and 10–28 and the PACAP fragment 6–38 do not act as agonists in the inhibition of IL-2, IL-6, and IL-10 (7, 13, 14). We first investigated the specificity of the inhibitory effect of VIP and PACAP on LPS-induced TNF-α release by comparing the effects of PACAP27, secretin, glucagon, and the VIP and PACAP fragments. TNF-α production by macrophages was inhibited by VIP, PACAP27, and PACAP38 to similar levels at all concentrations assayed (10^{-7}–10^{-11} M; Fig. 2*A*), whereas secretin, glucagon, $PACAP_{6-38}$, and the two VIP fragments failed to inhibit TNF-α production (Fig. 2*A*). These results confirm the specificity of the VIP and PACAP inhibitory activity, and the lack of effect of the

Table I. *Inhibitory effects of VIP and PACAP38 on TNF-α release by peritoneal macrophages stimulated with LPS*[a]

Treatment	Maximum Inhibition (%)[b]	IC_{50}(pM)[c]
VIP	57.0 ± 7.1	79.5 ± 3.28
PACAP	56.6 ± 5.2	81.1 ± 2.65

[a] Peritoneal macrophages (1×10^6 cells/ml) were stimulated with LPS (10 ng/ml) in the absence (control) or presence of VIP or PACAP (10^{-7}–10^{-13} M). The supernatants were collected after an incubation period of 6 h, and TNF-α levels were determined by ELISA. Control TNF-α levels were 1430 ± 97 pg/ml. Data are expressed as mean ± SD of six experiments performed in duplicate.

[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 six separate experiments.

neuropeptide fragments suggests that intact VIP and PACAP molecules are required for their inhibitory effect.

To determine which class of VIP/PACAP receptors is responsible for the inhibition of TNF-α production by VIP and PACAP, experiments using receptor antagonists were performed. We investigated the ability of $PACAP_{6-38}$, a PACAP antagonist with specificity for PAC1, and VPAC2 (15), and of [Ac-Tyr^1,D-Phe^2]GRF_{1-29} amide, a VPAC1 antagonist (16), to reverse the effects of VIP and PACAP on TNF-α production by incubating increasing concentrations of these VIP/PACAP antagonists (10^{-5}–10^{-8} M) in the presence of a fixed concentration of VIP or PACAP (10^{-8} M). The inhibitory effect of VIP and PACAP on TNF-α production was reversed by the VPAC1 antagonist in a dose-dependent manner (Fig. 2*B*). The most effective antagonist concentration was 10^{-5} M, which almost completely abolished the inhibitory effect of VIP and PACAP (Fig. 2*B*). A second, recently developed (17), VPAC1 antagonist [Ac-His^1,D-Phe^2,K^{15},R^{16},L^{27}]VIP_{3-7}-GRF_{8-27} had a similar effect (Fig. 2*C*). In contrast, the PAC1 antagonist did not reverse the inhibitory effect of VIP and PACAP (Fig. 2*B*). Neither the VPAC1 antagonists nor $PACAP_{6-38}$ significantly affected cell number or viability (data not shown) or TNF-α levels (Fig. 2). In addition, a newly described (18) VPAC1 agonist [K^{15},R^{16},L^{27}]VIP_{1-7}-GRF_{8-27} inhibited in a

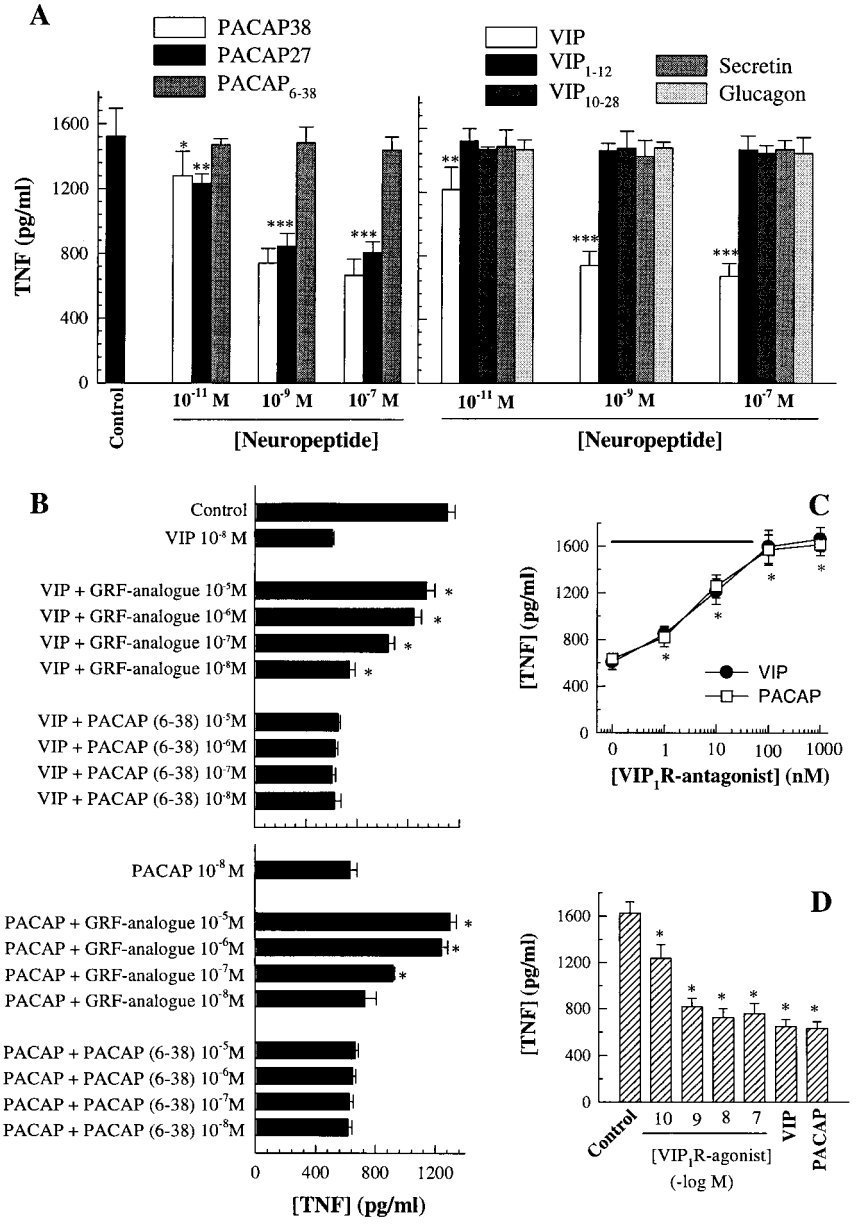

FIGURE 2. Inhibition of TNF-α production by VIP and PACAP is specific. *A*, Comparative effects of VIP, PACAP38, VIP-related peptides, and VIP and PACAP fragments on TNF-α production by LPS-stimulated macrophages. Cells (5×10^5 cells/ml) were stimulated with LPS (10 ng/ml) in the presence or the absence of three different concentrations of VIP, PACAP38, secretin, glucagon, VIP_{1-12}, VIP_{10-28}, PACAP27, and $PACAP_{6-38}$ (10^{-7}, 10^{-9}, and 10^{-11} M). Supernatants were collected 6 h later and were assayed for TNF-α production by ELISA. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (vs control cultures with LPS alone). *B* and *C*, Effects of PAC1 and VPAC1 antagonists on the inhibitory activity of VIP and PACAP on LPS-induced TNF-α production. Peritoneal macrophages (5×10^5 cells/ml) were stimulated with LPS (10 ng/ml) and were treated simultaneously with VIP or PACAP (10^{-8} M) and different concentrations of two VPAC1 antagonists, GRF analogue (*B*) and [Ac-His^1,D-Phe^2,K^{15},R^{16},L^{27}]VIP_{3-7}-GRF_{8-27} (*C*), or a PAC1-antagonist ($PACAP_{6-38}$). Supernatants were collected 6 h later and assayed for TNF-α production. TNF-α production by unstimulated and LPS-stimulated macrophages was, respectively, 69 ± 4 and 1479 ± 132 for 10^{-5} M GRF analogue, 66 ± 5 and 1468 ± 98 pg/ml for 10^{-5} M VPAC1 antagonist, and 65 ± 7 and 1482 ± 103 pg/ml for 10^{-5} M $PACAP_{6-38}$. The solid horizontal line (*C*) represents control values from cultures incubated with LPS alone (1621 ± 122 pg TNF-α/ml). Each result is the mean ± SD of four to six experiments performed in duplicate. *, $p < 0.001$ (vs samples treated with neuropeptides and without antagonists). *D*, Effect of a VPAC1 agonist on TNF-α production by macrophages. Macrophages (5×10^5 cells/ml) were stimulated with LPS (10 ng/ml) and treated with different concentrations of a newly synthesized VPAC1 agonist, [K^{15},R^{16},L^{27}]VIP_{1-7}-GRF_{8-27}. Supernatants were collected 6 h later and assayed for TNF-α production. Results are the mean ± SD of six experiments performed in duplicate. *, $p <$ 0.001 (vs controls (LPS alone).

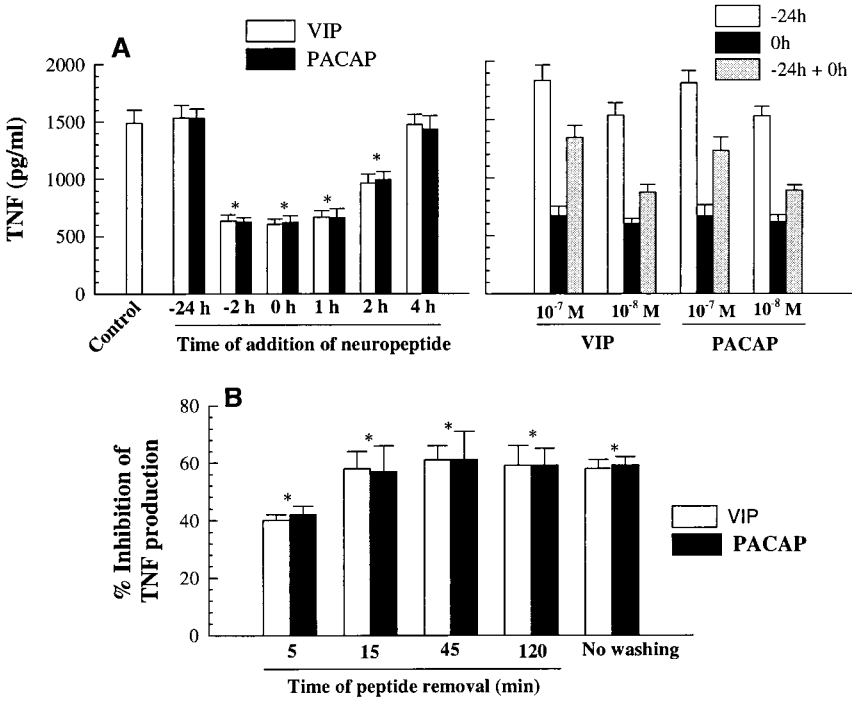

FIGURE 3. Time course for the VIP and PACAP inhibitory activity on LPS-activated TNF-α production. *A*, Effects of treating macrophages with VIP or PACAP before or after LPS stimulation. *Left*, Macrophages (5×10^5 cells/ml) were incubated with LPS (10 ng/ml) at time zero. VIP or PACAP (10^{-8} M) was added at various times relative to LPS as indicated. *Right*, VIP or PACAP (10^{-8} M) was added to macrophages (5×10^5 cells/ml) 24 h before LPS (10 ng/ml), with LPS (time zero), or at both time points. Supernatants were collected 6 h later after the initiation of the cultures (time zero) and were assayed for TNF-α 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 the absence of VIP or PACAP (10^{-8} M). Both neuropeptides were removed a different times by washing (three times with serum-free medium), and the cells were then resuspended in complete medium containing LPS (10 ng/ml; without neuropeptides) and cultured for an additional 6 h. Control cultures containing only LPS were washed and recultured under the same conditions. Supernatants were collected, and the TNF-α content was determined by ELISA. The percent inhibition was calculated by comparing each experimental culture with its control. Each result is the mean ± SD of four experiments performed in duplicate. ∗, $p < 0.001$ (vs LPS-stimulated controls without neuropeptides).

dose-dependent manner TNF production by macrophages, similar to VIP and PACAP (Fig. 2*D*). These results confirm the specificity of the VIP and PACAP inhibitory activity and suggest that both neuropeptides exert their actions through binding to VPAC1.

Time course for the inhibitory effect of VIP and PACAP on LPS-induced TNF-α production

In the experiments described above, VIP and PACAP were added to cells at the same time as LPS. We next investigated the effect of exposing macrophages to VIP or PACAP before and after LPS stimulation. We stimulated peritoneal macrophages with LPS (10 ng/ml) and added 10^{-8} M VIP or PACAP at different times before (24 and 2 h) and after (from 0–4 h) initiation of the cultures. Supernatants were collected 6 h after the initiation of cultures and were assayed for TNF-α production. The addition of VIP and PACAP up to 1 h after LPS stimulation resulted in significant levels of inhibition (57–60%; Fig. 3*A*). Later additions resulted in progressively lower degrees of inhibition (Fig. 3*A*). On the contrary, preincubation with VIP or PACAP for 24 h did not inhibit the latter LPS-stimulated TNF-α production. However, addition of VIP or PACAP to the cells 2 h before the addition of LPS inhibited TNF production as effectively as simultaneous addition (Fig. 3*A*). Furthermore, the higher concentration (10^{-7} M) of both neuropeptides prevented the suppression of TNF production by a second dose of VIP or PACAP added 24 h later (Fig. 3*B*). Preincubation with 10^{-8} M VIP or PACAP did not significantly prevent inhibition of TNF production by a second dose added to the cells with the LPS (Fig. 3*B*).

To determine the minimum time required for the inhibitory effect of VIP and PACAP, peritoneal macrophages were cultured with LPS in the presence or the absence of 10^{-8} M VIP or PACAP. Neuropeptides were removed by extensive washing at different times (from 5–120 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 resulted in significant inhibitory effects (Fig. 3*C*).

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

Next, to investigate whether the observed inhibition by VIP and PACAP was specific for endotoxin-induced TNF release or whether these neuropeptides might also act as inhibitors of TNF-α release by cytokine-activated macrophages, the effects of VIP and PACAP on IL-1β-induced TNF-α production were determined. VIP and PACAP attenuated the release of TNF-α by macrophages activated with 100 U/ml IL-1β, but to a greater extent than LPS-induced release (Fig. 4). The maximal VIP/PACAP inhibitory effect on cytokine-induced TNF-α production was exerted at 12 h (Fig. 4).

VIP and PACAP reduce TNF-α mRNA levels

Having demonstrated that VIP and PACAP had an inhibitory effect on endotoxin-induced TNF-α production, we determined whether

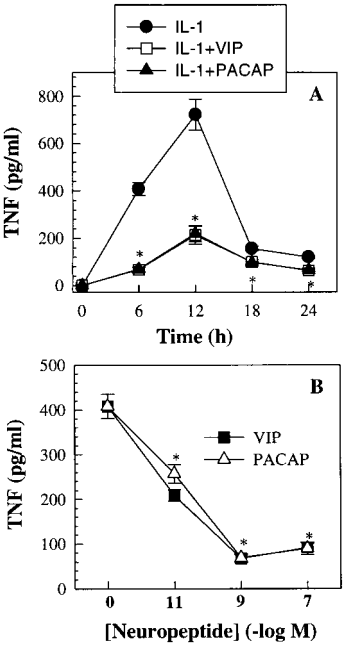

FIGURE 4. Modulation of IL-1β-induced TNF-α release by VIP and PACAP. *A*, Macrophages (5×10^5 cells/ml) were incubated with mrIL-1β (100 U/ml) in the absence or the presence of VIP or PACAP (10^{-8} M) for 24 h. At the indicated time points, supernatants were collected, and TNF-α release was determined by ELISA. *B*, Macrophages (5×10^5 cells/ml) were incubated with mrIL-1β (100 U/ml) in the absence or the presence of different concentrations of VIP or PACAP. The supernatants were collected 6 h later, and TNF-α release was determined by ELISA. Results are shown as the mean $\pm$ SD of three experiments performed in duplicate. Asterisks indicate a statistically significant difference compared with controls containing IL-1β in the absence of neuropeptides ($p < 0.001$).

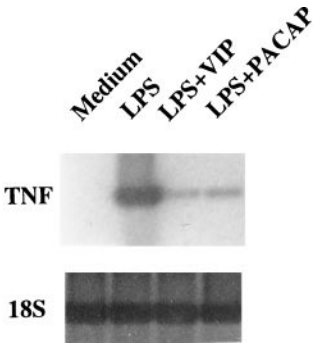

FIGURE 5. Inhibitory effects of VIP and PACAP on LPS-induced TNF-α mRNA expression. Macrophages (10^7 cells) were stimulated with LPS (10 ng/ml) and incubated without or with VIP or PACAP (10^{-8} M) for 3 h. Total RNA was extracted and blotted onto nylon membranes. The expression of TNF-α and 18S mRNA in peritoneal macrophages was analyzed by Northern blot analysis. One representative experiment of three is shown.

this action occurs at a transcriptional level. We stimulated peritoneal macrophages with LPS in the presence or the absence of 10^{-8} M VIP or PACAP. Total RNA was prepared from 3-h cultures and subjected to Northern blot analysis. Although no TNF-α mRNA was detectable in unstimulated cells (data not shown), a high level of TNF-α mRNA was present in LPS-stimulated macrophages, and the hybridization signal was strongly reduced in the presence of either VIP or PACAP (Fig. 5). These results indicate that VIP and PACAP could inhibit TNF-α production at the transcriptional level in LPS-stimulated macrophage cultures; however, it remains to be established whether the diminution in steady state TNF-α mRNA levels results from a decrease in either the novo transcriptional rate or message stabilization, or both.

Intracellular signal pathways involved in the inhibition of TNF-α production by VIP and PACAP

To study the second messengers involved in the inhibitory activity of VIP and PACAP on TNF-α release, we determined the effects of PMA (an activator of PKC), calphostin C (a specific inhibitor of PKC) (19), forskolin (an inducer of intracellular cAMP levels) (20), and H89 (a potent and specific inhibitor of PKA) (21) on

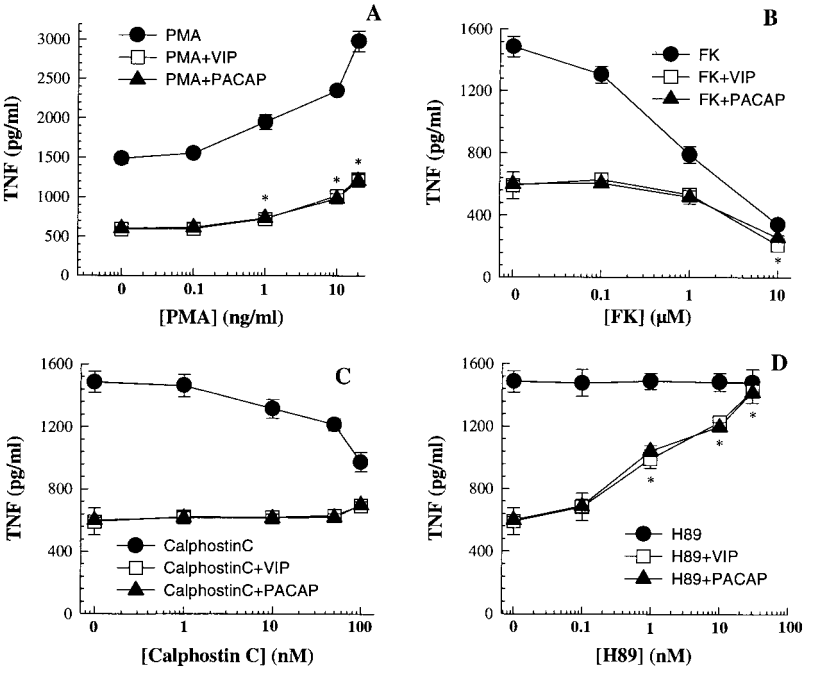

FIGURE 6. Comparative effects of PMA (PKC activator), forskolin (PKA activator), calphostin C (PKC inhibitor), and H89 (PKA inhibitor) on LPS-induced TNF-α production in murine macrophages incubated with VIP or PACAP. Peritoneal macrophages (5×10^5 cells/ml) were stimulated with LPS (10 ng/ml), LPS plus VIP (10^{-8} M), or LPS plus PACAP (10^{-8} M) in the absence or the presence of different concentrations of PMA (*A*), forskolin (FK; *B*), calphostin C (*C*), or H89 (*D*). Supernatants were collected 6 h later and assayed for TNF-α production by ELISA. Each result is the mean $\pm$ SD of three to six experiments performed in duplicate. *, $p < 0.001$ with respect to neuropeptide-treated samples without protein kinase modulators.

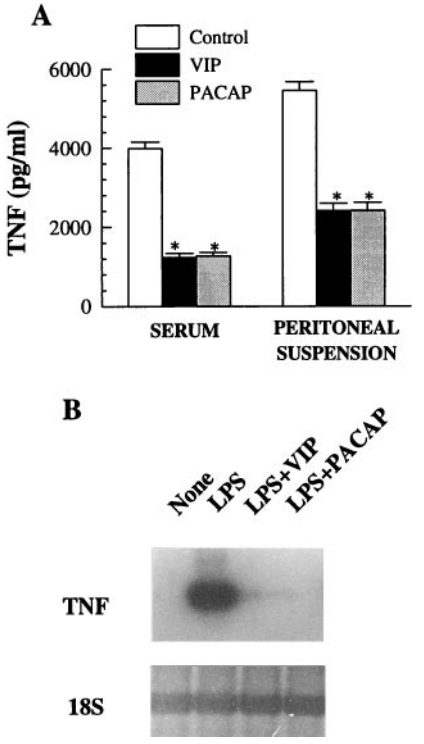

FIGURE 7. In vivo effects of VIP and PACAP on LPS-induced TNF-α release and mRNA expression. Mice were i.p. injected with LPS (25 μg/mouse), LPS plus VIP (5 nmol/mouse), or LPS plus PACAP (5 nmol/mouse). *A*, After 2 h, serum and peritoneal exudate were obtained as described in *Materials and Methods* and were assayed for TNF-α production by ELISA. Each result is the mean ± SD of three experiments performed in duplicate. ∗, $p < 0.001$ (vs control samples without neuropeptides). *B*, Expression of TNF-α and 18S mRNA in the peritoneal exudate was analyzed by Northern blot analysis 1.5 h post-LPS. Mice injected i.p. with medium alone were included (None). One representative experiment of three is shown.

TNF-α production by LPS-stimulated macrophages incubated in the absence or the presence of VIP or PACAP.

PMA induced TNF-α production in a dose-dependent manner, and VIP/PACAP inhibited the PMA-induced release of TNF-α (Fig. 6*A*). These results suggests that PKC plays a role in TNF-α production by macrophages. Indeed, calphostin C inhibited in a dose-dependent manner the TNF-α production by LPS-stimulated macrophages (Fig. 6*C*).

In terms of the secondary messenger involved in the inhibitory activity of VIP/PACAP, several experiments suggest the involvement of intracellular cAMP. For example, forskolin, which increases cAMP, inhibited TNF-α production in LPS-stimulated macrophages, similar to VIP/PACAP (Fig. 6*B*). The treatment with forskolin in addition to VIP/PACAP resulted in an additive effect only at the highest forskolin concentration used (10 μM; Fig. 6*B*). The fact that the effect of VIP/PACAP was not additive with forskolin suggests that both neuropeptides and forskolin mediate their actions through the same intracellular pathway. The involvement of cAMP in the inhibitory effect of VIP/PACAP on TNF-α production is also supported by the results obtained with the two specific inhibitors, i.e., calphostin C and H89. As reported above, calphostin C inhibited TNF-α release by LPS-stimulated macrophages, but did not reverse the inhibitory effect of VIP/PACAP (Fig. 6*C*). In contrast, whereas H89 does not affect TNF-α production by LPS-stimulated macrophages, it reverses the inhibitory effect of VIP/PACAP (Fig. 6*D*). These results suggest that whereas TNF-α production by LPS-stimulates macrophages involves PKC, the inhibitory effect of VIP/PACAP is exerted at least partially through increases in intracellular cAMP.

VIP and PACAP inhibit LPS-induced TNF-α production in vivo

An attempt was made to reproduce the in vitro observations in vivo. To this end, mice were treated with VIP, PACAP, or vehicle control solution, followed by the administration of LPS (from *S. enteridis*; 25 μg/mouse). The three experiments depicted in Fig. 7*A* show that both neuropeptides (a single dose of 5 nmol/mouse) decreased the concentration of TNF-α induced by LPS in serum and extracellular fluid (peritoneal lavage). Next, we used the same experimental protocol to analyze TNF-α mRNA in freshly peritoneal cells by Northern blot analysis. As shown in Fig. 7*B*, VIP and PACAP significantly reduced the expression of TNF-α mRNA after 1.5 h of endotoxin treatment.

In vitro and in vivo VIP production induced by LPS and proinflammatory cytokines

Finally, we investigated the effects of LPS and some proinflammatory cytokines involved in endotoxic shock, such as IL-1β, IL-6, and TNF-α, on VIP production by peritoneal cells. As shown in Fig. 8*A*, unstimulated peritoneal cells cultured in vitro release VIP, with maximum release at 24–36 h. Treatment of cultures with LPS, IL-1β, IL-6, or TNF-α resulted in a significant augmentation of the in vitro VIP production compared with that in the unstimulated controls. This stimulatory effect occurred over a period of 12–72 h of exposure to these endotoxemic mediators, with maximum levels at 24 h (Fig. 8*A*). At all times, LPS was the strongest activator of VIP production. The potencies of IL-1β, IL-6, and TNF-α were similar (Fig. 8*A*). Anti-mTNF-α or anti-mIL-6 Abs partially reversed the stimulatory effect of LPS on VIP production (Fig. 8*B*), and cotreatment with both anti-mTNF-α and anti-mIL-6 was clearly additive relative to treatment with either of these Abs, although the blockage was not total (Fig. 8*B*). Finally, the LPS-induced VIP production was corroborated in vivo, because LPS (25 μg/mouse) increased in a time-dependent manner VIP levels in the peritoneal suspension and serum, showing a maximum effect at 12 h (Fig. 8*C*). Interestingly, basal VIP levels were significantly higher in thioglycolate-elicited mice than in unstimulated mice (Fig. 8*C*). These results indicate that VIP production in vitro and in vivo is induced by LPS at least partially through the release of the proinflammatory cytokines TNF-α, IL-1β, and IL-6.

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 have been shown to attenuate reperfusion injury following ischemia of brain (22) and lung (23). VIP has a protective effect on the acute lung inflammation caused by several inflammatory agents (24–26). In this study we investigated the effects of VIP and PACAP on the production of TNF-α, a proinflammatory cytokine, by endotoxin-activated macrophages. Our findings show that VIP and PACAP inhibit in a similar way and in a time- and dose-dependent manner the LPS-stimulated production of TNF-α by murine macrophages.

The time course indicates that, similar to IL-6 and IL-10 (7, 14), the effect of VIP and PACAP on TNF-α production occurs rapidly, as 5- to 15-min incubations with either neuropeptide are sufficient to achieve a maximum effect. In addition, the inhibition has a refractory period of 2–3 h, after which the inhibitory activity of VIP or PACAP is reduced significantly, suggesting that VIP/PACAP block an early event in TNF-α generation in macrophages. The failure of VIP and PACAP to inhibit TNF-α production in

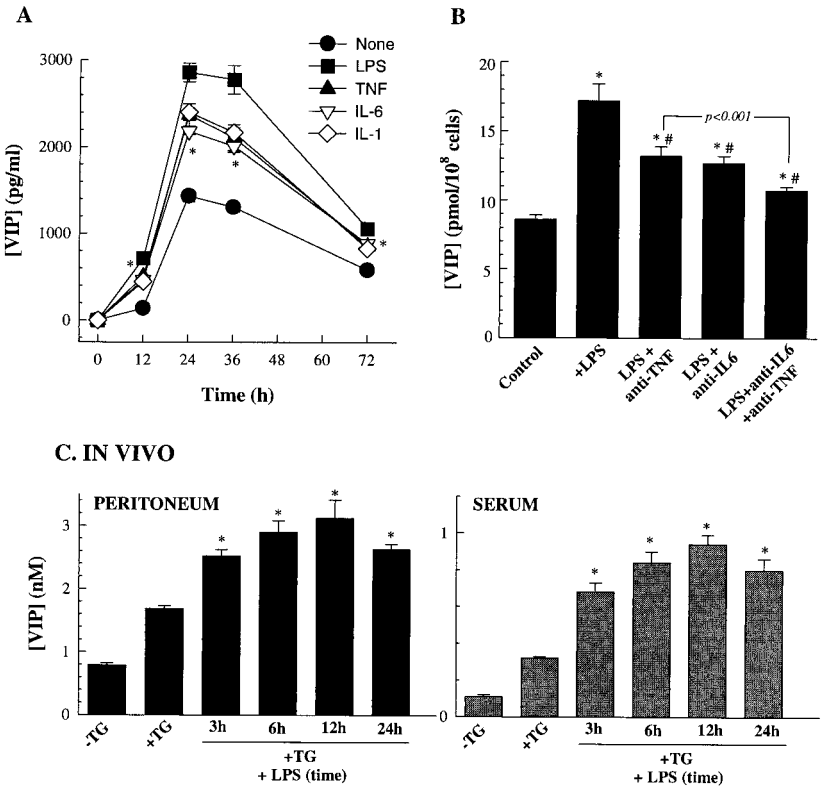

FIGURE 8. Effects of LPS, TNF-α, IL-6, and IL-1β on VIP production in murine peritoneal cells. *A*, In vitro time curve for LPS-, TNF-α-, IL-6-, and IL-1β-induced VIP release in peritoneal suspension. Fresh peritoneal cell suspension (5×10^6 cells/ml) was cultured in the absence (controls) or the presence of LPS (5 μg/ml), TNF-α (20 ng/ml), IL-6 (20 ng/ml), or IL-1β (10 ng/ml) for 72 h. At the indicated time points VIP production was assayed by ELISA as described in *Materials and Methods*. Each result is the mean ± SD of three experiments performed in duplicate. *, $p < 0.001$ (vs control samples without LPS/cytokines). *B*, LPS-induced VIP production is partially reversed by anti-TNF-α and anti-IL-6 Abs. Peritoneal cells (5×10^6 cells/ml) were incubated with LPS (5 μg/ml) in the presence or the absence of anti-mTNF-α Ab (50 μg/ml) and/or anti-mIL-6 Ab (50 μg/ml). Supernatants were collected 24 h later and assayed for VIP production by ELISA. Each result is the mean ± SD of three to five experiments performed in duplicate. *, $p < 0.001$ (vs control samples without LPS). #, $p < 0.001$ (vs samples treated with LPS alone, without Abs). *C*, Effect of LPS on in vivo production of VIP. Mice were injected i.p. with thioglycolate (+TG). After 4 days, mice were given an i.p. injection of LPS (25 μg/mouse), and at the indicated time points peritoneal and serum VIP levels were measured by ELISA as described in *Materials and Methods*. Basal VIP levels were determined in freshly peritoneal suspension and serum from mice without thioglycolate treatment (−TG). *, $p < 0.001$ (vs control samples without LPS). Each result is the mean ± SD of three experiments performed in duplicate.

response to delayed LPS stimulation could be explained by down-regulation of VIP receptors, which is known to occur in the presence of VIP (27).

Similar to the effect on other cytokines, such as IL-2, IL-6, and IL-10 (7, 13, 14), the inhibition of TNF-α requires intact VIP/PACAP molecules. This is in agreement with previous reports showing that either C- or N-terminal truncations of VIP lead to significant losses in biological activity (28, 29). The nature of the macrophage VIP/PACAP receptors that mediate the inhibition of TNF-α production is not clear. Peritoneal macrophages were previously shown to express PAC1 and VPAC1 mRNA, and both high affinity and low affinity VIP/PACAP binding sites were identified on peritoneal macrophages (4, 5, 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 inhibitory effect in our study. To confirm the specificity of the observed effects and to provide additional evidence for the receptor-mediated nature of the effects of VIP and PACAP, we investigated the ability of the $PACAP_{6-38}$ fragment, an antagonist with specificity for both the PAC1 and VPAC2 (15), and of two VPAC1 antagonists (16, 17) to reverse the inhibitory effect of VIP and PACAP on TNF-α production. The lack of effect of $PACAP_{6-38}$ eliminates PAC1 and VPAC1 as possible mediators. In contrast, the two VPAC1 antagonists are fully active in reversing the inhibitory effect of both VIP and PACAP, suggesting that this receptor is a viable candidate. This was confirmed by the fact that a VPAC1 agonist (18) showed a similar effect as VIP/PACAP by inhibiting TNF-α production.

The VPAC1 is coupled primarily to the adenylate cyclase system in several immune cells, including peritoneal macrophages (30–32). To fully understand the mechanism of action of VIP and PACAP, it is important to clarify which transduction pathways are involved in the inhibition of TNF-α production by macrophages. It has been shown that TNF-α production is inhibited by agents that increase intracellular cAMP levels and is stimulated by activation of the PKC pathway (1, 33–35). In the present study, using selective activators and inhibitors of PKA and PKC, we demonstrated that VIP and PACAP inhibit TNF-α production by macrophages through PKA activation and elevation of cAMP levels following binding to VIP_1R.

In a recent study we reported that in contrast to the broad inhibitory effects of VIP and PACAP on LPS-induced IL-6 release, both neuropeptides were ineffective in reducing production of IL-6 by macrophages stimulated with either IL-1β or TNF-α; on the contrary, we found that VIP and PACAP increased IL-6 levels (7).

However, in the present study we have shown that both neuropeptides inhibited in a dose-dependent manner IL-1β-induced TNF-α production. Similar results have been previously described in human monocytes for another anti-inflammatory substance, the endogenous nucleoside adenosine (36). This discrepancy between the inhibitory effects of VIP/PACAP and adenosine on IL-1β-induced TNF-α release on one side and the potentiating effect on the release of IL-6 on the other side, further argues against a common inhibitory mechanism for both cytokines.

Our previous experiments regarding VIP modulation of cytokine expression indicated that the inhibitory effects of VIP on IL-2/IL-6/IL-10 and IL-4 production result from different molecular mechanisms, i.e., transcriptional vs post-transcriptional regulation, respectively (6, 7). The present study indicates that, similar to IL-2, IL-6, and IL-10 and in contrast to IL-4, the inhibitory effect of VIP and PACAP on TNF-α production occurs through a transcriptional mechanism. In this sense we have recently reported that VIP and PACAP inhibit TNF-α transcriptional activation in LPS-stimulated Raw 264.7 cells by affecting both NF-κB nuclear translocation and the composition of the cAMP response element binding complex (CREB/c-Jun) (37).

The in vitro inhibitory effect of VIP/PACAP on LPS-induced TNF-α production correlates with the in vivo inhibition of both TNF-α expression and release in endotoxemic mice. 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 (4), and Gaytan et al. found PACAP immunoreactivity in lymphocytes from different lymphoid organs (38). In the present study we have demonstrated that LPS stimulation induced in vivo and in vitro VIP production and release by peritoneal cells (possibly lymphocytes). In addition, several proinflammatory cytokines, such as IL-1β, IL-6, and TNF-α, stimulated VIP synthesis in the peritoneal microenvironment. The fact that the LPS-induced VIP production is partially reversed by the addition of Abs against these proinflammatory cytokines suggests that the stimulatory action of LPS on VIP release is mediated at least partially through the LPS-induced synthesis of IL-1β, IL-6, and TNF-α by peritoneal macrophages. In this sense, several lines of evidence suggest that the production and expression of VIP and VIP binding sites are regulated by inflammatory mediators, such as IL-1, IL-6, TNF-α, antigenic stimulation, and glucocorticoids (39-44). These findings suggest the existence of a negative feedback, in which mediators produced by inflammatory cells following endotoxin stimulation stimulate VIP and VIP-R expression, and the subsequent VIP/VIP-R interactions negatively regulate the local inflammatory response. Therefore, the in vivo effect of VIP and PACAP on TNF-α 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.

There is abundant evidence that TNF-α is an important mediator of shock and organ failure complicating Gram-negative sepsis (1). Could the administration of VIP/PACAP early in sepsis, before the development of hypotension, be useful? In this sense, elevated VIP levels were reported in patients with Gram-negative septic shock and in some endotoxic animal models (45–49). We have recently demonstrated that VIP and PACAP protect mice from lethal endotoxemia through the inhibition of TNF-α and IL-6 production (50). In addition, an anti-shock effect of PACAP on experimental endotoxin shock in dogs has been previously reported (51). We propose that during a normal immune response, the timely production and/or release of VIP and possibly PACAP within the lymphoid organs following antigenic stimulation serve to downregulate the ongoing immune response, mostly through the modulation of cytokine production. During septic shock, however, due to severe septicemia leading to an overstimulation of the immune system, the effect of checkpoint molecules such as IL-10, IL-13, VIP, and PACAP is overwhelmed by the proinflammatory network. Based on the present study demonstrating that VIP and PACAP inhibit TNF-α production and on the protective effect of these anti-inflammatory neuropeptides in lethal endotoxic shock, the exogenous administration of VIP or PACAP could offer an alternative to existing treatments for septic shock syndrome.

Acknowledgments

We thank Dr. P. Robberecht for the kind gift of VIP_1R antagonist and agonist, and Dr. H. C. Wong for the kind gift of anti-VIP mAb.

References

1. Tracey, K. J., and A. Cerami. 1993. Tumor necrosis factor, other cytokines and disease. *Annu. Rev. Cell. Biol. 9:317.*
2. De la Fuente, M., M. Delgado, and R. P. Gomariz. 1996. VIP modulation of immune cell functions. *Adv. Neuroimmunol. 6:75.*
3. Segura, J. J., J. M. Guerrero, R. Goberna, and J. R. Calvo. 1991. Characterization of functional receptors for vasoactive intestinal peptide (VIP) in rat peritoneal macrophages. *Regul. Pept. 33:133.*
4. Delgado, M., D. Pozo, C. Martinez, E. Garrido, J. Leceta, J. R. Calvo, and R. P. Gomariz. 1996. Characterization of gene expression of VIP and VIP1-receptor in rat peritoneal lymphocytes and macrophages. *Regul. Pept. 62:161.*
5. Pozo, D., M. Delgado, C. Martinez, R. P. Gomariz, J. M. Guerrero, and J. R. Calvo. 1997. Functional characterization and mRNA expression of pituitary adenylate cyclase-activating polypeptide (PACAP) type I receptors in rat peritoneal macrophages. *Biochim. Biophys. Acta 1359:250.*
6. Ganea, D. 1996. Regulatory effects of vasoactive intestinal peptide on cytokine production in central and peripheral lymphoid organs. *Adv. Neuroimmunol. 6:61.*
7. Martinez, C., M. Delgado, D. Pozo, J. Leceta, J. R. Calvo, D. Ganea, and R. P. Gomariz. 1998. Vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide modulate endotoxin-induced IL-6 production by murine peritoneal macrophages. *J. Leukocyte Biol. 63:591.*
8. Platzer, C., C. Meisel, K. Vogt, M. Platzer, and H. D. Volk. 1995. Upregulation of monocytic IL-10 by tumor necrosis factor-α and cAMP elevating drugs. *Int. Immunol. 7:517.*
9. Engelberts, I., A. Moller, G. J. M. Schoen, C. J. Van der Linden, and W. A. Buurman. 1991. Evaluation of measurement of human TNF in plasma by ELISA. *Lymphokine Cytokine Res. 10:69.*
10. Fransen, L., R. Mueller, A. Marmenout, J. Tavernier, J. Van der Heyden, E. Kawashima, A. Collet, R. Tizard, H. Van Heuverswyn, A. Van Vliet, et al. 1985. Molecular cloning of mouse tumor necrosis factor cDNA and its eukaryotic expression. *Nucleic Acids Res. 13:4417.*
11. Gomariz, R. P., E. Garrido, J. Leceta, C. Martinez, R. Abalo, and M. Delgado. 1994. Gene expression of VIP receptor in rat lymphocytes. *Biochem. Biophys. Res. Commun. 203:1599.*
12. Tatsuno, I., P. E. Gottschall, and A. Arimura. 1991. Inhibition of mitogen-stimulated proliferation of murine splenocytes by a novel neuropeptide, pituitary adenylate cyclase-activating polypeptide: a comparative study with vasoactive intestinal peptide. *Endocrinology 128:728.*
13. Xin, Z., H. Tang, and D. Ganea. 1994. Vasoactive intestinal peptide inhibits interleukin (IL)-2 and IL-4 production in murine thymocytes activated via TCR/CD3 complex. *J. Neuroimmunol. 54:59.*
14. Martinez, C., M. Delgado, R. P. Gomariz, and D. Ganea. 1996. Vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide-38 inhibit IL-10 production in murine T lymphocytes. *J. Immunol. 156:4128.*
15. Gourlet, P., M. C. Vandermeers-Piret, J. Rathe, P. De Neef, and P. Robberecht. 1995. Fragments of pituitary adenylate cyclase-activating polypeptide discriminate between type I and II recombinant receptors. *Eur. J. Pharmacol. 287:7.*
16. Waelbroeck, M., P. Robberecht, D. H. Coy, J. Camus, P. De Neef, and J. Christophe. 1995. Interaction of growth hormone-releasing factor (GRF) and 14 GRF analogs with vasoactive intestinal peptide (VIP) receptors of rat pancreas: discovery of (N-Ac-Tyr^1,D-Phe^2)-GRF (1–29)-NH_2 as a VIP antagonist. *Endocrinology 116:2643.*
17. Gourlet, P., P. De Neef, J. Cnudde, M. Waelbroeck, and P. Robberecht. 1997. In vitro properties of a high affinity selective antagonist of the VIP_1 receptor. *Peptides 18:1555.*
18. Gourlet, P., A. Vandermeers, P. Vertongen, J. Ratche, P. De Neef, J. Cnudde, M. Waelbroeck, and P. Robberecht. 1997. Development of high affinity selective VIP_1 receptor agonists. *Peptides 18:1539.*
19. Kobayashi, E., H. Nakno, M. Morimoto, and T. Tamaoki. 1989. Calphostin C (UCN-1028C), a novel microbial compound, is a highly potent and specific inhibitor of protein kinase C. *Biochem. Biophys. Res. Commun. 159:548.*

20. Boige, N., B. Aminaroff, A. Munk, and M. Laburthe. 1984. Forskolin stimulates adenylate cyclase in human colonic crypts: interaction with VIP. *Eur. J. Pharmacol. 101:111.*
21. Chijiwa, T., A. Mishima, M. Hagiwara, M. Sano, K. Hayashi, T. Inoue, K. Naito, T. Toshioka, and H. Hidaka. 1990. Inhibition of forskolin-induced neurite outgrowth and protein phosphorylation by a newly synthesized selective inhibitor of cyclic AMP-dependent protein kinase, *N*-[2-(*p*-bromociannamylamino)ethyl]-5-isoquinoleinsulfonamide (H89), of PC12D pheochromocytoma cells. *J. Biol. Chem. 265:5267.*
22. Uchida, D., A. Arimura, A. Somogyvri-Vigh, S. Shioda, and W. A. Banks. 1996. Prevention of ischemia-induced death of hippocampal neurons by pituitary adenylate cyclase activating polypeptide. *Brain Res. 736:280.*
23. Said, S. I. 1985. Vasoactive intestinal peptide. In *Airways Smooth Muscle: Peptide Receptors, Ion Channels and Signal Transduction.* D. Raeburn and M. A. Giembycz, eds. Birkhauser Verlag, Basel, p. 87.
24. Foda, H. D., T. Iwanaga, L. W. Liu, and S. I. Said. 1988. Vasoactive intestinal peptide protects against HCl-induced pulmonary edema in rats. *Ann. NY Acad. Sci. 527:633.*
25. Berisha, H., H. D. Foda, H. Sakakibara, D. Trotz, H. Pakbaz, and S. I. Said. 1990. Vasoactive intestinal peptide prevents lung injury due to xanthine/xanthine oxidase. *Am. J. Physiol. 259:L151.*
26. Kurosawa, M., and T. Ishizuka. 1993. Inhibitory effects of vasoactive intestinal peptide on superoxide anion formation by *N*-formyl-methionyl-leucyl-phenylalanine-activated inflammatory cells in vitro. *Int. Arch. Allergy Appl. Immunol. 100:28.*
27. Pozo, D., J. R. Calvo, and J. M. Guerrero. 1995. Homologous regulation of vasoactive intestinal peptide (VIP) receptors on rat peritoneal macrophages. *Peptides 16:313.*
28. Gourlet, P., J.-P. Vilardaga, P. DeNeef, M. Waelbroeck, A. Vandermeers, and P. Robberecht. 1996. The C-terminus ends of secretin and VIP interact with the N-terminal domains of their receptors. *Peptides 17:825.*
29. O'Donnell, M., R. J. Garippa, N. C. O'Neill, D. R. Bolin, and J. M. Cottrell. 1991. Structure-activity studies of vasoactive intestinal polypeptide. *J. Biol. Chem. 266:6389.*
30. Calvo, J. R., D. Pozo, and J. M. Guerrero. 1996. Functional and molecular characterization of VIP receptors and signal transduction in human and rodent immune systems. *Adv. Neuroimmunol. 6:39.*
31. Calvo, J. R., M. L. Montilla, J. M. Guerrero, and J. J. Segura. 1994. Expression of VIP receptors in mouse peritoneal macrophages: functional and molecular characterization. *J. Neuroimmunol. 50:85.*
32. Pozo, D., J. J. Segura, I. Carrero, L. G. Guijarro, J. C. Prieto, and J. R. Calvo. 1996. Characterization of adenylyl cyclase stimulated by VIP in rat and mouse peritoneal macrophages membranes. *Biochim. Biophys. Acta 1312:249.*
33. Kovacs, E. J., D. Radzioch, H. A. Young, and L. Varesio. 1988. Differential inhibition of IL-1 and TNF-α mRNA expression by agents which block second messenger pathways in murine macrophages. *J. Immunol. 141:3101.*
34. Severn, A., N. T. Rapson, C. A. Hunter, and F. Y. Liew. 1992. Regulation of tumor necrosis factor production by adrenaline and β-adrenergic agonists. *J. Immunol. 148:3441.*
35. Renz, H., J. H. Gong, A. Schmitd, M. Nain, and D. Gemsa. 1988. Release of tumor necrosis factor-α from macrophages: enhancement and suppression are dose-dependently regulated by prostaglandin E_2 and cyclic nucleotides. *J. Immunol. 141:2388.*
36. Bouma, M. G., R. K. Stad, F. A. J. M. Van den Wildenberg, and W. A. Buurman. 1994. Differential regulatory effects of adenosine on cytokine release by activated human monocytes. *J. Immunol. 153:4159.*
37. Delgado, M., E. J. Munoz-Elias, Y. Kan, I. Gozes, M. Fridkin, D. E. Brenneman, R. P. Gomariz, and D. Ganea. 1999. Vasoactive intestinal peptide and pituitary adenylate cyclase activating polypeptide inhibit TNFα transcriptional activation by regulating NF-κB and CREB/c-Jun. *J. Biol. Chem. 273:31427.*
38. Gaytan, F., A. J. Martinez-Fuentes, F. Gracia-Navarro, H. Vaudry, and E. Aguilar. 1994. Pituitary adenylate cyclase-activating polypeptide (PACAP) immunolocalization in lymphoid tissues of the rat. *Cell Tissue Res. 276:223.*
39. Baumann, H., A. J. Symes, M. R. Comeau, K. K. Morella, Y. Wang, D. Friend, S. F. Ziegler, J. S. Fink, and D. P. Gearing. 1994. Multiple regions within the cytoplasmic domains of the leukemia inhibitory factor receptor and gp130 cooperate in signal transduction in hepatic and neuronal cells. *Mol. Cell. Biol. 14:138.*
40. Ferguson, T. A., S. Fletcher, J. Herndon, and T. S. Griffith. 1995. Neuropeptides modulate immune deviation induced via the anterior chamber of the eye. *J. Immunol. 155:1746.*
41. Rao, M. S., Y. Sun, U. Vaidyanathan, S. C. Landis, and R. E. Zigmond. 1993. Regulation of substance P is similar to that vasoactive intestinal peptide after axotomy or explantation of the rat superior cervical ganglion. *J. Neurobiol. 24: 571.*
42. Eskay, R. L., and L. E. Eiden. 1992. Interleukin-1α and tumor necrosis factor-α differentially regulate enkephalin, vasoactive intestinal peptide, neurotensin, and substance P biosynthesis in chromaffin cells. *Endocrinology 130:2252.*
43. Kaltreider, H. B., S. Ichikawa, P. K. Byrd, D. A. Ingram, J. L. Kishiyama, S. P. Sreedharan, M. L. Warnock, J. M. Beck, and E. J. Goetzl. 1997. Upregulation of neuropeptides and neuropeptide receptors in a murine model of immune inflammation in lung parenchyma. *Am. J. Respir. Cell Mol. Biol. 16:133.*
44. Segura, J. J., J. M. Guerrero, D. Pozo, J. R. Calvo. 1996. Expression of vasoactive intestinal peptide binding sites in rat peritoneal macrophages is stimulated by inflammatory stimulus. *J. Neuroimmunol. 64:1.*
45. Brandtzaeg, P., O. Oktedalen, P. Kierulf, and P. K. Opstad. 1989. Elevated VIP and endotoxin plasma levels in human Gram-negative septic shock. *Regul. Pept. 24:37.*
46. Higashiguchi, T., Y. Noguchi, A. Noffsinger, J. E. Fischer, P. O. Hasselgren. 1994. Sepsis increases production of total secreted proteins, vasoactive intestinal peptide, and peptide YY in isolated rat enterocytes. *Am. J. Surg. 168:251.*
47. Zamir, O., P. O. Hasselgren, T. Higashiguchi, J. A. Frederick, and J. E. Fischer. 1992. Effect of sepsis or cytokine administration on release of gut peptides. *Am. J. Surg. 163:181.*
48. Revhaug, A., I. Lygren, T. I. Lundgren, O. K. Andersen, P. G. Burhol, and K. E. Giercksky. 1985. Increased plasma levels of vasoactive intestinal peptide in pigs during endotoxemia. *Eur. Surg. Res. 17:75.*
49. Broner, C. W., M. S. O'Dorisio, R. B. Rosenberg, and T. M. O'Dorisio. 1995. Cyclic nucleotides and vasoactive intestinal peptide production in a rabbit model of *Escherichia coli* septicemia. *Am. J. Med. Sci. 309:267.*
50. Delgado, M., C. Martinez, D. Pozo, J. R. Calvo, J. Leceta, D. Ganea, and R. P. Gomariz. 1999. Vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide protect mice from lethal endotoxemia through the inhibition of TNF-α and IL-6. *J. Immunol. 162,* In press.
51. Suzuki, Y., K. Kasai, I. Lino, K. Takekoshi, M. Oka, and S. Shimoda. 1994. Anti-shock effect of pituitary adenylate cyclase activating polypeptide (PACAP) on experimental endotoxin shock in dogs. *Life Sci. 54:389.*