

# Vasoactive Intestinal Peptide and Pituitary Adenylate Cyclase-Activating Polypeptide Prevent Inducible Nitric Oxide Synthase Transcription in Macrophages by Inhibiting NF- $\kappa$ B and IFN Regulatory Factor 1 Activation<sup>1</sup>

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High-output nitric oxide (NO) production from activated macrophages, resulting from the induction of inducible NO synthase (iNOS) expression, represents a major mechanism for macrophage cytotoxicity against pathogens. However, despite its beneficial role in host defense, sustained high-output NO production was also implicated in a variety of acute inflammatory diseases and autoimmune diseases. Therefore, the down-regulation of iNOS expression during an inflammatory process plays a significant physiological role. This study examines the role of two immunomodulatory neuropeptides, the vasoactive intestinal peptide (VIP) and the pituitary adenylate cyclase-activating polypeptide (PACAP), on NO production by LPS-, IFN- $\gamma$ -, and LPS/IFN- $\gamma$ -stimulated peritoneal macrophages and the Raw 264.7 cell line. Both VIP and PACAP inhibit NO production in a dose- and time-dependent manner by reducing iNOS expression at protein and mRNA level. VPAC1, the type 1 VIP receptor, which is constitutively expressed in macrophages, and to a lesser degree VPAC2, the type 2 VIP receptor, which is induced upon macrophage activation, mediate the effect of VIP/PACAP. VIP/PACAP inhibit iNOS expression and activity both in vivo and in vitro. Two transduction pathways appear to be involved, a cAMP-dependent pathway that preferentially inhibits IFN regulatory factor-1 transactivation and a cAMP-independent pathway that blocks NF- $\kappa$ B binding to the iNOS promoter. The down-regulation of iNOS expression, together with previously reported inhibitory effects on the production of the proinflammatory cytokines IL-6, TNF- $\alpha$ , and IL-12, and the stimulation of the anti-inflammatory IL-10, define VIP and PACAP as "macrophage deactivating factors" with significant physiological relevance. *The Journal of Immunology*, 1999, 162: 4685–4696.

Nitric oxide (NO)<sup>3</sup> is an unstable free radical gas that mediates many physiological and toxic functions, such as macrophage cytotoxicity, neurotransmission, neurotoxicity, and regulation of blood pressure (1). The nitric oxide synthase (NOS) family of enzymes that catalyze the conversion of L-arginine to NO and L-citrulline can be classified into two major groups: constitutive NOS and inducible NOS (iNOS). Neuronal NOS and endothelial NOS are in general constitutively expressed and dependent on high intracellular Ca<sup>2+</sup> levels (2). Macrophages express a transcriptionally inducible form of NOS (iNOS), independent of elevated Ca<sup>2+</sup> and undetectable unless the cells are activated (2). Typically, a synergistic combination of stimuli is required for maximal induction of iNOS mRNA. For murine mac-

rophages, IFN- $\gamma$  and LPS represent one of the most potent combinations of synergizing stimuli (3). Once synthesized, iNOS is responsible for prolonged, high-output production of NO. Induced NO production is one of the principal mechanisms of macrophage cytotoxicity for tumor cells, bacteria, protozoa, helminthes, and fungi (1, 4). In general, expression of iNOS follows a generalized or localized inflammatory response resulting from infection or tissue injury. Despite its beneficial role in host defense, sustained NO production can be deleterious to the host, and NO synthesis induced by cytokines and/or inflammatory stimuli has been implicated in experimental arthritis, inflammatory bowel disease, hypotension associated with septic shock, and other types of tissue injury (5–8). Therefore, the selective inhibition of expression of this enzyme represents an important therapeutic goal.

Vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating polypeptide (PACAP) are two multifunctional neuropeptides whose primary immunomodulatory function is anti-inflammatory in nature. VIP and PACAP have been shown to attenuate reperfusion injury following ischemia of brain and lung (8–10), to inhibit T cell proliferation and cytokine production (reviewed in Ref. 11), and to inhibit several macrophage functions, such as phagocytosis, respiratory burst, chemotaxis (reviewed in Ref. 12), and LPS-induced IL-6 and TNF- $\alpha$  production (13–16). Furthermore, we have recently demonstrated that VIP and PACAP protect mice from lethal endotoxemia, presumably by reducing the levels of endogenous TNF- $\alpha$  and IL-6 (17).

The functional relationship between VIP and NO is rather complicated. VIP and NO can be colocalized and coreleased from some neurons and can regulate each other's release in some tissues (reviewed in Ref. 8). In some physiological outcomes, such as the relaxation of smooth muscle, VIP and NO cooperate, whereas in

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<sup>3</sup> Abbreviations used in this paper: NO, nitric oxide; IRF-1, IFN regulatory factor-1; EMSA, electrophoretic mobility shift assay; NOS, nitric oxide synthase; iNOS, inducible nitric oxide synthase; nNOS, neuronal nitric oxide synthase; PAC1, pituitary adenylate cyclase-activating polypeptide receptor; PACAP, pituitary adenylate cyclase-activating polypeptide; PKA, protein kinase A; VIP, vasoactive intestinal peptide; VPAC1, type 1 vasoactive intestinal peptide receptor; VPAC2, type 2 vasoactive intestinal peptide receptor.

others, such as the inflammatory response, they play opposite roles, with NO increasing and VIP defending against tissue and cell injury. The opposite role played by VIP and NO in an inflammatory process raises the possibility that VIP may regulate NO production or activity. Indeed, in injury models that involve neuronal NOS, VIP does not inhibit NO synthesis, but prevents its toxic action (18, 19). However, much less is known about the role of VIP in the expression or activity of iNOS, the macrophage-specific NOS, in response to inflammation.

To further clarify the role played by VIP and PACAP in the attenuation of the inflammatory response, in this study we examine the *in vitro* and *in vivo* effects of both neuropeptides on NO production and iNOS transcription in activated peritoneal macrophages. We investigate the molecular mechanisms involved, including the specific receptors, the intracellular signal pathways, and the nuclear transactivating factors that mediate the effect of VIP/PACAP on NO synthesis.

## Materials and Methods

### Reagents

Synthetic VIP, PACAP38, VIP<sub>1-12</sub>, and VIP<sub>10-28</sub> were purchased from Novabiochem (Laufelfingen, Switzerland). The type 1 VIP receptor (VPAC1) antagonist [Ac-His<sup>1</sup>, D-Phe<sup>2</sup>, K<sup>15</sup>, R<sup>16</sup>, L<sup>27</sup>] VIP (3-7)-GRF (8-27) and the VPAC1 agonist [K<sup>15</sup>, R<sup>16</sup>, L<sup>27</sup>] VIP (1-7)-GRF (8-27) were kindly donated by Dr. Patrick Robberecht (Universite Libre de Bruxelles, Belgium). The VPAC2 agonist Ro 25-1553 Ac-[Glu<sup>8</sup>, Lys<sup>12</sup>, Nle<sup>17</sup>, Ala<sup>19</sup>, Asp<sup>25</sup>, Leu<sup>26</sup>, Lys<sup>27,28</sup>, Gly<sup>29,30</sup>, Thr<sup>31</sup>]-VIP cyclo (21-25) was a generous gift from Drs. Ann Welton and David R. Bolin (Hoffmann-La Roche, Nutley, NJ). The synthetic PACAP receptor (PAC1) agonist maxadilan was a generous gift from Dr. Ethan A. Lerner (Massachusetts General Hospital, Charlestown, MA). The PAC1 antagonist PACAP<sub>6-38</sub>, secretin, and glucagon were obtained from Peninsula Laboratories (Belmont, CA). Oligonucleotides were synthesized by the Oligonucleotide Synthesis Service from Rutgers University (Newark, NJ). Murine recombinant IFN- $\gamma$  was purchased from PharMingen (San Diego, CA). LPS (from *Escherichia coli* 055:B5), calphostin C, forskolin, prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), L-arginine, NADPH, flavin-adenine dinucleotide, tetrahydrobiopterin, L-lactic dehydrogenase, sodium pyruvate, sulfanilamide, *N*-[naphthyl]ethyl-enediamine dihydrochloride, protease inhibitors, PMSF, EDTA, glycine, protein G-agarose, glycerol, EGTA, and DTT were purchased from Sigma (St. Louis, MO), and *N*-[2-(*p*-bromocinnamyl-amino) ethyl]-5-iso-quinolinesulfonamide (H89) from ICN Pharmaceuticals (Costa Mesa, CA). Abs against IFN regulatory protein-1 (IRF-1) and NF- $\kappa$ B (p50 and p65) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

### Cell cultures

Mouse peritoneal macrophages were elicited by *i.p.* injection of 2 ml of 4% Brewer's thioglycollate medium (Difco, Detroit, MI) into male BALB/c mice (aged 6-10 wk). Peritoneal exudate cells were obtained 72 h after injection by peritoneal lavage with ice-cold RPMI 1640 medium. Peritoneal exudate cells, containing lymphocytes and macrophages, were washed twice and resuspended in ice-cold RPMI 1640 medium supplemented with 2% heat-inactivated FCS (Life Technologies, Grand Island, NY), containing 10 mM HEPES buffer, 1 mM pyruvate, 0.1 M nonessential amino acids, 2 mM glutamine, 50 mM 2-ME, 100 U/ml penicillin, and 10  $\mu$ g/ml streptomycin (RPMI 1640 complete medium). Cells were seeded in flat-bottom 96-well microtiter plates (Corning Glass, Corning, NY) at  $8 \times 10^4$  cells per well in a final volume of 200  $\mu$ l. The cells were incubated at 37°C for 2 h to allow adherence to plastic, and nonadherent cells were removed by repeated washing with RPMI 1640 medium. At least 96% of the adherent cells were macrophages as judged by morphologic and phagocytic criteria.

Raw 264.7 mouse macrophage cells (American Type Culture Collection, Manassas, VA) were cultured in DMEM supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 10  $\mu$ g/ml streptomycin, and 10% FCS (complete culture medium). The cells were plated in flat-bottom 96-well microtiter plates for 24 h. Nonadherent cells were removed by aspiration and two washings with DMEM medium.

Macrophage monolayers (murine peritoneal macrophages and Raw 264.7 cells) were incubated with complete medium and stimulated with 0.5  $\mu$ g/ml LPS or/and 200 U/ml IFN- $\gamma$  in the presence or absence of VIP or PACAP38 (from  $10^{-12}$  to  $10^{-7}$  M) at 37°C in a humidified incubator

with 5% CO<sub>2</sub>. Cell-free supernatants were harvested at the designated time points and kept frozen (-20°C) until NO determination.

### Determination of NO

The amount of NO formed was estimated from the accumulation of the stable NO metabolite nitrite by the Griess assay (20). Equal volumes of culture supernatants (90  $\mu$ l) and Griess reagents (90  $\mu$ l of 1% sulfanilamide/0.1% *N*-[naphthyl]ethyl-enediamine dihydrochloride in 2.5% H<sub>3</sub>PO<sub>4</sub>) were mixed, and the absorbance was measured at 550 nm. The amount of nitrite was calculated from a NaNO<sub>2</sub> standard curve.

### Determination of NO synthase activity

Lysates from activated macrophages were prepared, and the NO synthase activity was measured as described (21). Murine peritoneal macrophages and Raw 264.7 cells were activated with IFN- $\gamma$ /LPS in the presence or absence of VIP or PACAP and harvested at 4°C. Cells were lysed by three cycles of freeze/thaw in 40 mM Tris-HCl, pH 8.0, 0.1 mM EGTA, 0.1 mM EDTA, 12 mM 2-ME, and protease inhibitors (0.1 mM PMSF, 5  $\mu$ g/ml aprotinin, 1  $\mu$ g/ml leupeptin, 1  $\mu$ g/ml chymostatin, and 5  $\mu$ g/ml pepstatin), and centrifuged at 10,000  $\times g$  for 60 min. For the NO synthase assay, macrophage lysates were incubated with 2 mM L-arginine, 2 mM NADPH, 4  $\mu$ M flavin-adenine dinucleotide, 4  $\mu$ M tetrahydrobiopterin, 2 mM DTT, and 1 mM EGTA for 2 h at 37°C. The reaction was terminated by adding 15 U/ml L-lactic dehydrogenase and 83 mM sodium pyruvate and incubating for an additional 15 min. Nitrite concentration in the reaction mixture was measured as described above.

In addition, iNOS activity was also evaluated by measuring arginine to citrulline conversion as previously described (22). Briefly, macrophage monolayers were cultured in 12-well plates ( $4 \times 10^6$  cells/well in 2 ml of complete RPMI medium) in the presence of LPS/IFN- $\gamma$  and various concentrations of VIP or PACAP for 24 h. The cells were washed twice in medium, and [<sup>3</sup>H]arginine (3  $\mu$ Ci/ml) (Amersham, Arlington Heights, IL) was added for 20 min. Incorporation of radiolabeled arginine was terminated by rapid aspiration of the extracellular medium and two washes with ice-cold PBS. The cells were lysed by the addition of 1 ml of 0.4 N HClO<sub>4</sub>, and the intracellular extract was neutralized with K<sub>2</sub>CO<sub>3</sub> and 50 mM Tris/HCl buffer, pH 7.4. Aliquots (100  $\mu$ l) of the neutralized intracellular extract were used to measure [<sup>3</sup>H]arginine uptake. To separate [<sup>3</sup>H]citrulline from [<sup>3</sup>H]arginine, extract aliquots of 400  $\mu$ l were applied to 1-ml Dowex AG50WX-8 (Na<sup>+</sup> form) columns as previously described (23). The columns were eluted with three volumes of distilled water, and the radioactivity in the flow-through fractions (containing almost exclusively [<sup>3</sup>H]citrulline) was quantitated by scintillation spectroscopy. All measurements were made in duplicate, corrected for the number of counts per  $4 \times 10^6$  cells, and the data are presented as recovery of radiolabeled citrulline.

### Western blot analysis

**iNOS.** Macrophage monolayers were washed three times with warm (37°C) PBS, harvested in cold 40 mM Tris/HCl buffer, pH 8, containing 5  $\mu$ g/ml aprotinin, 1  $\mu$ g/ml chymostatin, 50  $\mu$ g/ml leupeptin, 5  $\mu$ g/ml pepstatin, and 100  $\mu$ M PMSF, and lysed by sonication. For detection of iNOS by Western blotting, 25  $\mu$ g of macrophage lysates were separated on 7.5% SDS-PAGE gels under reducing conditions, transferred to reinforced nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany), and immunoblotted with anti-mouse iNOS mAb (Transduction Labs, Lexington, KY) as previously described (24).

**NF- $\kappa$ B and IRF-1.** Nuclear extracts were prepared from  $1 \times 10^7$  cells by resuspending the cells in 400  $\mu$ l of ice-cold 10 mM HEPES, pH 7.9, containing 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 10  $\mu$ g/ml aprotinin, 10  $\mu$ g/ml leupeptin, 10  $\mu$ g/ml pepstatin, and 1 mM sodium azide. After storage at 4°C for 10 min, Nonidet P-40 was added to a final concentration of 0.5%, the cells were vortexed gently for 15 s and centrifuged for 40 s at 12,000  $\times g$ . The pelleted nuclei were washed once with the above buffer and resuspended in 100  $\mu$ l of ice-cold 20 mM HEPES, pH 7.9, containing 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 25% glycerol, 1 mM DTT, 0.5 mM PMSF, 10  $\mu$ g/ml aprotinin, 10  $\mu$ g/ml leupeptin, 10  $\mu$ g/ml pepstatin, and 1 mM sodium azide. After incubation on ice for 30 min with vigorous vortexing every 10 min, the nuclear proteins were collected by centrifugation for 10 min at 12,000  $\times g$ . For detection of NF- $\kappa$ B and IRF-1, 25  $\mu$ g of nuclear proteins were separated on 7.5% SDS-PAGE gels under reducing conditions, transferred to nitrocellulose membranes, and immunoblotted with Abs against p50, p65, and IRF-1 (Santa Cruz Biotechnology) as recommended by the manufacturer.

### cAMP determinations

Following incubations for various times (from 0 to 120 min) with different concentrations of VIP, PACAP, or forskolin, macrophage monolayers ( $2 \times 10^6$  cells) were rinsed with ice-cold PBS and snap frozen in a dry ice-acetone bath. The cells were harvested in 1 ml of ice-cold 75% ethanol containing 0.5 mM 3-isobutyl-1-methylxanthine and centrifuged to remove the insoluble material. The supernatants were lyophilized and redissolved in 1 ml of 50 mM sodium acetate buffer, pH 6.2. The amounts of cAMP were quantitated by using a commercially available ELISA kit (Amersham).

### RNA extraction and Northern blot analysis

Northern blot analysis was performed according to standard methods. Macrophage monolayers ( $2 \times 10^6$  cells/ml) were stimulated with LPS (0.5  $\mu\text{g}/\text{ml}$ ) and IFN- $\gamma$  (200 U/ml), in the absence or presence of VIP and PACAP ( $10^{-8}$  M) for different time periods at 37°C. Total RNA was extracted by the acid guanidinium-phenol-chloroform method, electrophoresed on 1.2% agarose-formaldehyde gels, transferred to S&S Nytran membranes (Schleicher and Schuell, Keene, NJ), and cross-linked to the nylon membrane using UV light.

The probes for murine iNOS and  $\beta$ -actin were generated by RT-PCR as previously described (25, 26). Oligonucleotides were end-labeled with [ $\gamma$ - $^{32}\text{P}$ ]ATP by using T4 polynucleotide kinase. The RNA-containing membranes were prehybridized for 16 h at 42°C and then hybridized at 42°C for 16 h with the appropriate probes. The membranes were washed twice in  $2 \times \text{SSC}$  containing 0.1% SDS at room temperature (20 min each time), once at 37°C for 20 min, and once in  $0.1 \times \text{SSC}$  containing 0.1% SDS at 50°C (20 min). The prehybridization and hybridization buffers were purchased from 5 Prime  $\rightarrow$  3 Prime (Boulder, CO). The membranes were exposed to x-ray films (Kodak, Rochester, NY), and analyzed by densitometric analysis.

### In vivo quantitation of NO production and iNOS activity and expression

Male mice (6–10 wk old) were injected with a single dose of LPS (100  $\mu\text{g}/\text{mouse}$ ) i.p. in the presence or absence of different amounts of VIP or PACAP (0.5–10 nmol/mouse). At different time points (2–8 h), blood was removed through cardiac puncture, and peritoneal exudate was obtained as described above. The peritoneal suspensions were centrifuged for 5 min at  $1800 \times g$ , and cell-free supernatants were harvested. Serum and peritoneal cell-free supernatants were assayed for NO production. The peritoneal cells were subjected to Northern blot analyses and assayed for iNOS activity as described above.

### Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared by the miniextraction procedure of Schreiber et al. (27) with slight modifications. Raw 264.7 cells were plated at a density of  $10^7$  cells in six-well plates, stimulated, washed twice with ice-cold PBS/0.1% BSA, and scraped off the dishes. The cell pellets were homogenized with 0.4 ml of buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 10  $\mu\text{g}/\text{ml}$  aprotinin, 10  $\mu\text{g}/\text{ml}$  leupeptin, 10  $\mu\text{g}/\text{ml}$  pepstatin, and 1 mM  $\text{NaN}_3$ ). After 15 min on ice, Nonidet P-40 was added to a final 0.5% concentration, the tubes were gently vortexed for 15 s, and nuclei were sedimented and separated from cytosol by centrifugation at  $12,000 \times g$  for 40 s. Pelleted nuclei were washed once with 0.2 ml of ice-cold buffer A, and the soluble nuclear proteins were released by adding 0.1 ml of buffer C (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 25% glycerol, 1 mM DTT, 0.5 mM PMSF, 10  $\mu\text{g}/\text{ml}$  aprotinin, 10  $\mu\text{g}/\text{ml}$  leupeptin, 10  $\mu\text{g}/\text{ml}$  pepstatin, and 1 mM  $\text{NaN}_3$ ). After incubation for 30 min on ice, followed by centrifugation for 10 min at 14,000 rpm at 4°C, the supernatants containing the nuclear proteins were harvested, the protein concentration was determined by the Bradford method, and aliquots were stored at  $-80^\circ\text{C}$  for later use in EMSA.

Oligonucleotides corresponding to the  $\kappa\text{B}$  (nucleotides -92 to -65) and IRF-1 (nucleotides -933 to -906) motifs of the iNOS promoter were synthesized (28, 29): 5'-CCAAGTGGGACTCTCCCTTTGGGAACA-3' ( $\kappa\text{B}$ ), and 5'-CACTGTCAATATTTCACTTTCATAATG-3' (IRF-1). The oligonucleotides were annealed after incubation for 5 min at 85°C in 10 mM Tris-HCl, pH 8.0, 5 mM NaCl, 10 mM  $\text{MgCl}_2$ , and 1 mM DTT. Aliquots of 50 ng of the double-stranded oligonucleotides were end-labeled with [ $\gamma$ - $^{32}\text{P}$ ]ATP by using T4 polynucleotide kinase. For EMSA with macrophage nuclear extracts, 20,000–50,000 cpm of double-stranded oligonucleotides, corresponding to approximately 0.5 ng, were used for each reaction. The binding reaction mixtures (15  $\mu\text{l}$ ) were set up containing 0.5–1 ng DNA probe, 5  $\mu\text{g}$  nuclear extract, 2  $\mu\text{g}$  poly(dI-dC).poly(dI-dC), and binding buffer (50 mM NaCl, 0.2 mM EDTA, 0.5 mM DTT, 5% glycerol,

and 10 mM Tris-HCl pH 7.5). The mixtures were incubated on ice for 15 min before adding the probe, followed by another 20 min at room temperature. Samples were loaded onto 4% nondenaturing polyacrylamide gels and electrophoresed in TGE buffer (50 mM Tris-HCl, pH 7.5, 0.38 M glycine, and 2 mM EDTA) at 100 V, followed by transfer to Whatman paper, drying under vacuum at 80°C, and autoradiography. In competition and Ab supershift experiments, the nuclear extracts were incubated for 15 min at room temperature with the specific Ab (1  $\mu\text{g}$ ) or competing cold oligonucleotide (50-fold excess) before the addition of the labeled probe.

### Statistical analysis

All values are expressed as the mean  $\pm$  SD of the number of experiments. Each sample was assayed in duplicate. 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

### VIP and PACAP inhibit NO production

To determine the effect of VIP and PACAP on NO production, we first determined the amounts of NO following treatment of thioglycollate-elicited peritoneal macrophages with different stimuli. Macrophages were stimulated with LPS, IFN- $\gamma$ , or LPS plus IFN- $\gamma$ . Supernatants harvested 24 h later were assayed for the generation of nitrite, an accumulated oxidative product of NO. Unstimulated macrophages produce very low amounts of NO (Fig. 1A). Detectable levels of NO were present in macrophage cultures stimulated with all stimuli used, with the highest NO production observed upon stimulation with LPS plus IFN- $\gamma$  (Fig. 1A). VIP and PACAP inhibited NO production by  $\sim 95\%$  in macrophages stimulated with LPS, and by  $\sim 70\%$  and  $50\%$  in macrophages activated with IFN- $\gamma$  or LPS plus IFN- $\gamma$ , respectively (Fig. 1A). In addition, VIP and PACAP inhibited in a dose- and time-dependent manner the NO production by LPS- and LPS/IFN- $\gamma$ -stimulated cells (Fig. 1, B and C). The dose-response curves were similar for VIP and PACAP, showing maximal effects at  $10^{-8}$  M and an  $\text{IC}_{50}$  (i.e., the concentration of neuropeptide producing 50% of maximal inhibition) of  $\sim 0.50$  nM (Fig. 1) for both stimuli. The time curves indicate that the NO generation was significantly inhibited by VIP and PACAP as early as 16 h, with the maximum inhibitory effect after 24 h of culture (Fig. 1). Moreover, the reduction of NO generation was maintained throughout the 72-h incubation period (data not shown), indicating that VIP/PACAP do not delay, but rather reduce NO release.

The inhibitory effects were not the result of a decreased number of peritoneal macrophages, as neither VIP nor PACAP affected cell numbers or the viability of stimulated macrophages after 36 h of culture (viabilities were in the range of 89–97% with or without neuropeptides).

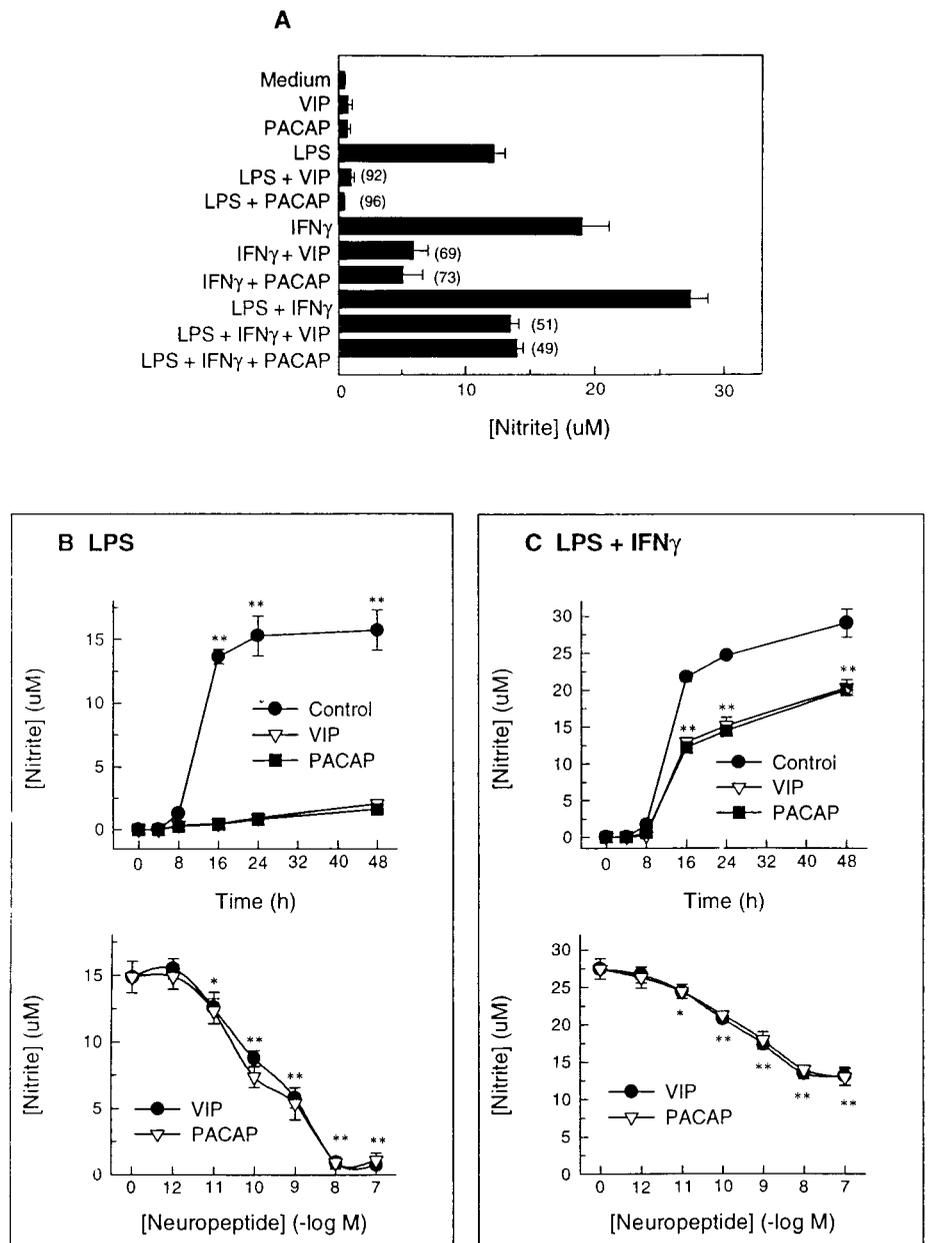
Optimal conditions were selected to further study the inhibitory effect of VIP and PACAP on NO production, i.e., macrophages were stimulated with LPS or LPS plus IFN- $\gamma$  in the presence of  $10^{-8}$  M neuropeptide for 24 h.

### VPAC1, and to a lesser degree VPAC2, mediate the inhibition of NO production by VIP and PACAP

Next we investigated whether the inhibitory effect of VIP/PACAP could be related to occupancy of specific receptors. First, we compared the effect of VIP/PACAP to that of secretin, glucagon, and the VIP and PACAP fragments VIP<sub>1–12</sub>, VIP<sub>10–28</sub>, and PACAP<sub>6–38</sub>. NO production was not affected by secretin and glucagon (Fig. 2A). The two VIP fragments and PACAP<sub>6–38</sub> failed to inhibit NO generation, suggesting that intact VIP and PACAP molecules are required for their inhibitory activity (Fig. 2A).

The immunological actions of VIP and PACAP are exerted through a family of receptors consisting of VPAC1, VPAC2, and PAC1 (30). Peritoneal macrophages express VPAC1 and PAC1

**FIGURE 1.** VIP and PACAP inhibit NO production by macrophages. *A*, Peritoneal macrophages were incubated with medium alone, LPS (500 ng/ml), IFN- $\gamma$  (200 U/ml), or LPS (500 ng/ml) plus IFN- $\gamma$  (200 U/ml) in the presence or absence of VIP or PACAP ( $10^{-8}$  M). Supernatants were collected after 24 h, and nitrite release was determined. Numbers in parenthesis represent percentage of inhibition calculated by comparison with corresponding controls without neuropeptides. *B* and *C*, Time course and dose-response curve for the inhibitory effect of VIP/PACAP on NO production. Macrophages were stimulated with LPS (500 ng/ml) (*B*) or LPS (500 ng/ml) and IFN- $\gamma$  (200 U/ml) (*C*) in the absence or presence of  $10^{-8}$  M VIP or PACAP (*upper panels*) or of a concentration range of either VIP or PACAP (*lower panels*). Supernatants collected at different times (*upper panels*) or 24 h (*lower panels*) were assayed for nitrite production. Control cultures were incubated with LPS (*B*) or LPS/IFN- $\gamma$  (*C*) alone. Each result is the mean  $\pm$  SD of five separate experiments performed in duplicate. \*,  $p < 0.05$ ; \*\*,  $p < 0.001$  with respect to control cultures treated with LPS or LPS/IFN- $\gamma$  alone.

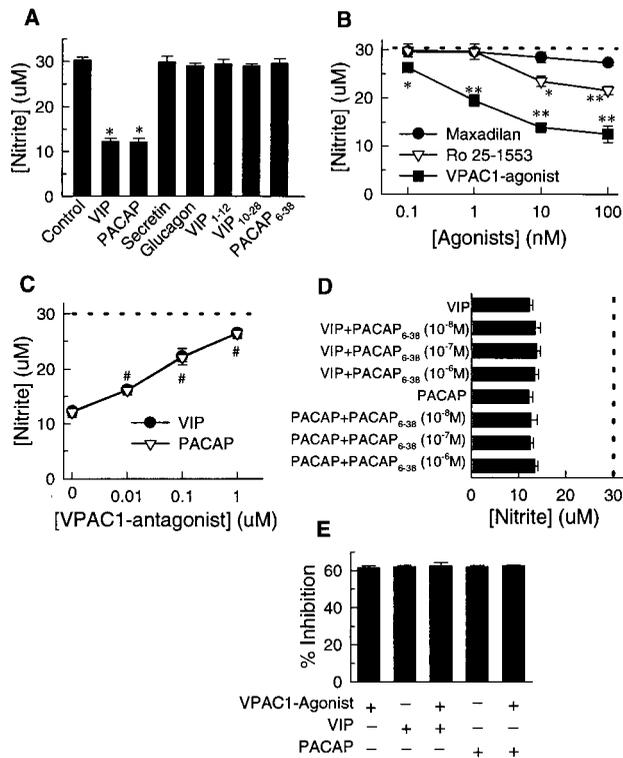


mRNA constitutively, and VPAC2 mRNA is inducible upon LPS stimulation (31, 32, 71). Similar results were obtained for the Raw 264.7 macrophage cell line (33). To determine which of the VIP/PACAP receptors are involved in the inhibition of NO production, we used specific receptor agonists and antagonists. We investigated the effect of a VPAC1 agonist (34), a VPAC2 agonist (Ro 25-1553) (35), and of maxadilan, a specific PAC1 agonist (36), on the LPS/IFN- $\gamma$ -induced NO production. VPAC1, and to a lesser degree VPAC2 agonists, but not the PAC1 agonist, inhibit NO release (Fig. 2B). The VPAC1 agonist exhibits a potency similar to that of VIP/PACAP (60% inhibition), whereas Ro 25-1553 was much less efficient (17–24% inhibition) (Fig. 2B). In addition, we investigated the ability of PACAP<sub>6–38</sub>, an antagonist specific for PAC1 and, to a lesser degree for VPAC2 (37), and of a specific VPAC1 antagonist (38), to reverse the effects of VIP and PACAP. Increasing concentrations of the antagonists ( $10^{-8}$  to  $10^{-6}$  M) were added simultaneously with a fixed concentration of VIP or PACAP ( $10^{-8}$  M). The inhibitory effects of VIP and PACAP were reversed by the VPAC1 antagonist in a dose-

dependent manner (Fig. 2C). In contrast, PACAP<sub>6–38</sub> did not reverse the inhibitory effect of VIP or PACAP (Fig. 2D). Neither the VPAC1 antagonist nor PACAP<sub>6–38</sub> significantly affected NO levels (Fig. 2, C and D). Furthermore, the simultaneous addition of VIP or PACAP and the VPAC1 agonist did not result in an additive effect on NO generation (Fig. 2E). Together these results confirm the specificity of the VIP and PACAP inhibitory activity, and suggest that both neuropeptides exert their action primarily through binding to VPAC1. Because VPAC2 appears to be expressed relatively late following LPS stimulation (12 and 24 h) (see Footnote 4), the lower efficiency of the VPAC2 agonist may result from a low number of VPAC2 receptors early during culture.

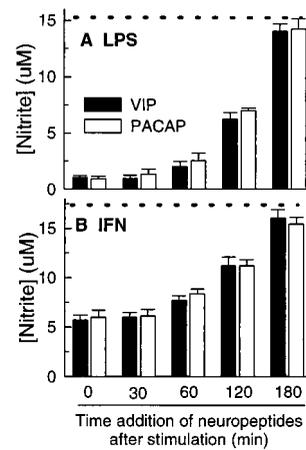
#### Effects of delayed addition of VIP and PACAP after macrophage activation

In the experiments described so far, VIP and PACAP were added to cells at the same time as LPS. To find the level of VIP/PACAP-induced blocking in NO generation, we next investigated the effect



**FIGURE 2.** Inhibition of NO production by VIP and PACAP is specific. *A* and *B*, Comparative effects of VIP, PACAP38, VIP-related peptides, VIP and PACAP fragments, and VIP and PACAP agonists on NO production. Peritoneal macrophages were stimulated with LPS (500 ng/ml) and IFN- $\gamma$  (200 U/ml) in the presence or absence of secretin, glucagon, VIP<sub>1-12</sub>, VIP<sub>10-28</sub>, and PACAP<sub>6-38</sub> ( $10^{-7}$  M) (*A*) or maxadilan (a PAC1 agonist), Ro 25-1553 (a VPAC2 agonist), and [K<sup>15</sup>, R<sup>16</sup>, L<sup>27</sup>]VIP (1-7)-GRF (8-27) (a VPAC1 agonist) (*B*). Supernatants were collected 24 h later and assayed for nitrite production. Each result is the mean  $\pm$  SD of five experiments performed in duplicate. \*,  $p < 0.001$  with respect to control cultures treated with LPS/IFN- $\gamma$  alone. *C* and *D*, Effect of PAC1 and VPAC antagonists on the inhibitory effect of VIP and PACAP. Peritoneal macrophages were stimulated with LPS (500 ng/ml) and IFN- $\gamma$  (200 U/ml), and treated simultaneously with VIP or PACAP ( $10^{-8}$  M) and different concentrations of a VPAC1 antagonist, [Ac-His<sup>1</sup>,D-Phe<sup>2</sup>, K<sup>15</sup>, R<sup>16</sup>, L<sup>27</sup>]VIP (3-7)-GRF (8-27) (*C*), or a PAC1/VPAC2 antagonist (PACAP<sub>6-38</sub>) (*D*). Supernatants were collected 24 h later and assayed for nitrite production. Nitrite production by LPS/IFN- $\gamma$ -stimulated cells was, respectively,  $30.15 \pm 1.23$   $\mu$ M for  $10^{-6}$  M VPAC1 antagonist and  $31.33 \pm 2.48$   $\mu$ M for  $10^{-6}$  M PACAP<sub>6-38</sub>. The dotted line represents control values from cultures incubated with LPS/IFN- $\gamma$  alone ( $30.17 \pm 2.41$   $\mu$ M). Each result is the mean  $\pm$  SD of four experiments performed in duplicate. #,  $p < 0.001$  compared with samples treated with neuropeptides and without antagonists. *E*, Effect of the VPAC1 agonist on NO production by VIP/PACAP. Macrophages were stimulated with LPS (500 ng/ml) and IFN- $\gamma$  (200 U/ml) and treated with the VPAC1 agonist (100 nM) in the presence or absence of VIP or PACAP ( $10^{-8}$  M). Supernatants were collected 24 h later and assayed for nitrite production. Percentage of inhibition was calculated by comparison with controls containing LPS/IFN- $\gamma$  alone. Results are the mean  $\pm$  SD of four experiments performed in duplicate.

of exposing macrophages to VIP or PACAP after LPS or IFN- $\gamma$  stimulation. We stimulated peritoneal macrophages with LPS (500 ng/ml) or with IFN- $\gamma$  (200 U/ml) and added  $10^{-8}$  M VIP or PACAP at different times after (from 0 to 3 h) the initiation of the cultures. Supernatants were collected 24 h after the initiation of the cultures and were assayed for NO production. The addition of VIP and PACAP up to 2 h after LPS or IFN- $\gamma$  stimulation resulted in significant levels of inhibition (50% for LPS stimulation and 32%



**FIGURE 3.** Effects of delayed addition of VIP and PACAP on NO production. Peritoneal macrophages were stimulated with LPS (500 ng/ml) (*A*) or IFN- $\gamma$  (200 U/ml) (*B*) at time 0. VIP or PACAP ( $10^{-8}$  M) were added at different times after the initiation of the cultures. Supernatants were collected 24 h after the initiation of the cultures and assayed for nitrite production. The dotted line represents control values from cultures incubated with LPS ( $15.24 \pm 1.11$   $\mu$ M) or IFN- $\gamma$  ( $17.23 \pm 1.54$   $\mu$ M) alone. Each result is the mean  $\pm$  SD of four experiments performed in duplicate.

for IFN- $\gamma$  stimulation) (Fig. 3). Later additions resulted in progressively lower degrees of inhibition (Fig. 3). Thus, VIP and PACAP block an early event in expression NO generation activity in macrophages.

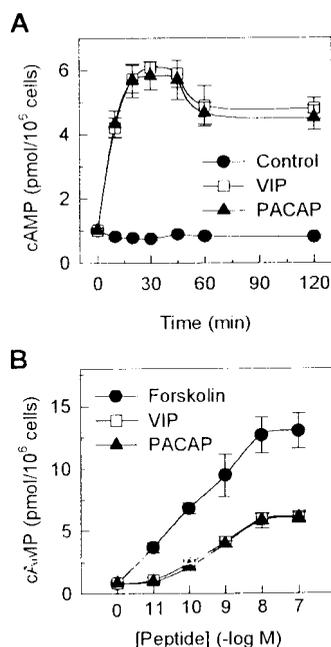
*Intracellular pathways involved in the inhibition of NO production by VIP and PACAP*

Because all three types of VIP/PACAP receptors induce cAMP in various cell types, and cAMP-inducing agents inhibit NO production (39), we measured first the amounts of cAMP generated in LPS/IFN- $\gamma$ -stimulated peritoneal macrophages after incubation with VIP or PACAP. VIP and PACAP increased, in a dose-dependent and time-dependent manner, the levels of intracellular cAMP (Fig. 4, *A* and *B*). However, the effect of the two neuropeptides was lower than that of forskolin (Fig. 4*A*).

To determine whether intracellular cAMP is involved in the inhibitory effect of VIP/PACAP on NO production, we investigated the effects of calphostin C (a protein kinase C inhibitor), H89 (a protein kinase A (PKA) inhibitor), and forskolin and PGE<sub>2</sub> (two strict cAMP-inducing agents). Forskolin and PGE<sub>2</sub> inhibit NO production in LPS/IFN- $\gamma$ -stimulated macrophages, although they show less of an effect at lower concentrations ( $10^{-8}$  and  $10^{-7}$  M) as compared with VIP and PACAP (Fig. 5*A*). In addition, the involvement of cAMP is supported by the results obtained with the two protein kinase inhibitors. In contrast to calphostin C, H89 partially reverses the inhibitory effect of VIP/PACAP (Fig. 5*B*). These results suggest that the inhibitory effect of VIP/PACAP is mediated, at least partially, through increases in intracellular cAMP.

*VIP and PACAP inhibit iNOS protein expression and iNOS activity*

Concentrations of VIP and PACAP shown to efficiently suppress the release of NO also reduce the expression of iNOS protein in LPS/IFN- $\gamma$ -stimulated macrophages (Fig. 6*A*). The reduction in iNOS is comparable in total peritoneal macrophage lysates, membrane and cytosol preparations (data not shown). The reduction in iNOS protein expression was paralleled by a decrease in iNOS



**FIGURE 4.** VIP and PACAP increase intracellular cAMP levels. Peritoneal macrophages were stimulated with LPS (0.5  $\mu\text{g/ml}$ ) and IFN- $\gamma$  (200 U/ml) in the presence or absence of VIP ( $10^{-8}$  M) or PACAP ( $10^{-8}$  M) (A) or of a VIP/PACAP concentration range (B). After 30 min (B), or at different times (A), intracellular cAMP was extracted and quantitated as described in *Materials and Methods*. Results are expressed as pmol cAMP/ $10^6$  cells. Each result is the mean  $\pm$  SD of three experiments performed in duplicate.

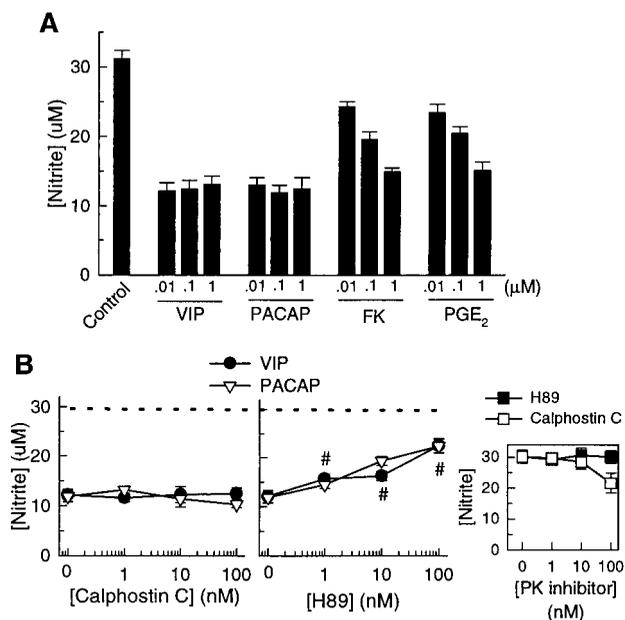
enzymatic activity, as measured by conversion of arginine to citrulline (Fig. 6B). VIP/PACAP also decreased iNOS activity in macrophage lysates supplemented with L-arginine and all cosubstrates and cofactors (data not shown). Therefore, the reduced accumulation of nitrite in VIP/PACAP-treated macrophage cultures reflects the decrease in iNOS protein, and is not a consequence of L-arginine or cosubstrate/cofactor depletion.

#### VIP and PACAP reduce iNOS mRNA levels

Having demonstrated that VIP and PACAP inhibit NO production, we sought to determine whether this action occurs at a transcriptional level through the inhibition of iNOS mRNA expression. We stimulated peritoneal macrophages with LPS plus IFN- $\gamma$  in the presence or absence of  $10^{-8}$  M VIP or PACAP for 4, 8, 16, and 24 h, and total RNA was prepared and subjected to Northern blot analysis. Although no iNOS mRNA is detectable in unstimulated cells (Fig. 7), progressively increased levels of iNOS mRNA are present in LPS/IFN- $\gamma$ -stimulated cells (up to 16 h) (Fig. 7). At all time points, VIP and PACAP significantly inhibited the levels of iNOS mRNA (Fig. 7), with a maximum effect at 16 h. The amount of iNOS mRNA correlated with the generation of nitrite in replica dishes (not shown). These results indicate that both neuropeptides inhibit iNOS steady-state mRNA levels.

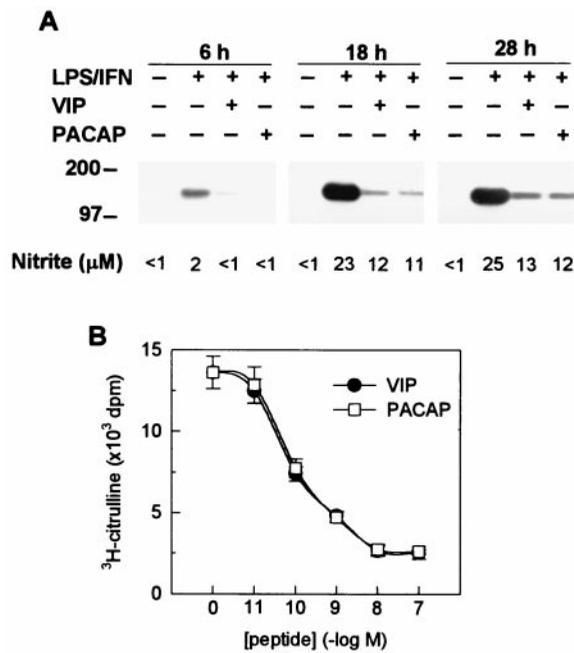
#### VIP and PACAP inhibit NF- $\kappa$ B and IRF-1 binding

Although the iNOS promoter contains a complex array of transactivating binding sites, NF- $\kappa$ B and IRF-1 appear to be essential for maximal iNOS transcription after LPS/IFN- $\gamma$  stimulation (28, 29, 40). To investigate whether VIP/PACAP affect NF- $\kappa$ B and IRF-1 binding, we used the murine macrophage cell line Raw 264.7. First, we confirmed that VIP and PACAP affect NO production in the Raw cells similar to peritoneal macrophages. In-



**FIGURE 5.** Intracellular signal pathways involved in the inhibitory activity of VIP and PACAP. A, Effect of cAMP-inducing agents. Peritoneal macrophages were stimulated with LPS (500 ng/ml) and IFN- $\gamma$  (200 U/ml) in the presence or absence of different concentrations of VIP, PACAP, forskolin (FK), or PGE<sub>2</sub>. Supernatants collected after 24 h were assayed for nitrite production. Control cultures were incubated with LPS/IFN- $\gamma$  alone. Each result is the mean  $\pm$  SD of five experiments performed in duplicate. B, Comparative effects of calphostin C (a protein kinase C-inhibitor) and H89 (a PKA-inhibitor). Peritoneal macrophages were stimulated with LPS (500 ng/ml) and IFN- $\gamma$  (200 U/ml), and incubated with or without VIP or PACAP ( $10^{-8}$  M), in the absence or presence of different concentrations of calphostin C or H89. Supernatants collected after 24 h were assayed for nitrite production. The dotted line represents control values from cultures incubated with LPS/IFN- $\gamma$  alone ( $29.76 \pm 1.68$   $\mu\text{M}$ ). Each result is the mean  $\pm$  SD of five experiments performed in duplicate. #,  $p < 0.001$  with respect to neuropeptide-treated samples without protein kinase inhibitors.

deed, VIP and PACAP inhibit, in a dose- and time-dependent manner, the NO production in LPS/IFN- $\gamma$ -stimulated Raw cells (Fig. 8A), with very similar kinetics to those observed in peritoneal macrophages. In addition, the two neuropeptides significantly reduce both iNOS activity and expression in Raw cells (Fig. 8A). The effects of VIP and PACAP on NF- $\kappa$ B and IRF-1 were studied by EMSA. Stimulation of Raw cells with LPS plus IFN- $\gamma$  led to a time-dependent increase in both NF- $\kappa$ B and IRF-1 binding compared with unstimulated cells; treatment with VIP and PACAP significantly inhibited the binding (Fig. 8, B and C, upper panels). The specificity of the NF- $\kappa$ B and IRF-1 binding was evident by the complete displacement of the NF- $\kappa$ B and IRF-1/DNA binding complexes in the presence of a 50-fold excess of unlabeled homologous oligonucleotides in the competition reactions (Fig. 8, B and C, upper panels). In contrast, a 50-fold excess of unlabeled nonhomologous oligonucleotides had no effects on this DNA binding activity (Fig. 8, B and C, upper panels). Ab supershift experiments were performed to determine the composition of the NF- $\kappa$ B and IRF-1-binding factors. Addition of either monospecific anti-p50 or anti-p65 Abs to the binding reaction resulted in a marked reduction in the intensity of the NF- $\kappa$ B band and led to the appearance of slow migrating bands, indicating that the NF- $\kappa$ B-binding complex is composed primarily of p50/p65 heterodimers (Fig. 8B, lower panel). Furthermore, the IRF-1/DNA complexes were supershifted by an anti-IRF-1 Ab in both LPS/IFN- $\gamma$ - and VIP/PACAP-treated cells (Fig. 8C, lower panel).

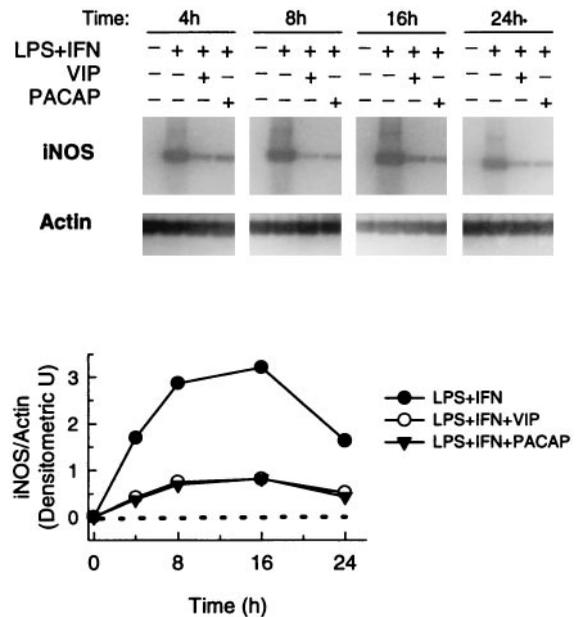


**FIGURE 6.** VIP and PACAP inhibit iNOS activity and protein expression. *A*, VIP and PACAP inhibit iNOS protein expression. Peritoneal macrophages were incubated with medium alone or stimulated with LPS (500 ng/ml) and IFN- $\gamma$  (200 U/ml) in the presence or absence of VIP or PACAP ( $10^{-8}$  M). At different time periods, the cells were harvested and lysed, and the lysates were subjected to Western blots as described in *Materials and Methods*. The nitrite concentrations in the culture supernatants are given below the respective lanes. The data shown are from one of three similar experiments. *B*, VIP and PACAP inhibit iNOS activity. Peritoneal macrophages were stimulated with LPS (500 ng/ml) and IFN- $\gamma$  (200 U/ml) in the presence or absence of different concentrations of VIP or PACAP for 24 h. The conversion of [ $^3\text{H}$ ]arginine to [ $^3\text{H}$ ]citrulline was used as a measure of iNOS activity. Disintegrations per minute for control unstimulated cultures averaged  $950 \pm 76$ . Data are the mean  $\pm$  SD of four experiments performed in duplicate.

#### Involvement of VPAC1 and cAMP in the effects of VIP on $\kappa\text{B}$ and IRF-1 binding

Because the inhibitory effect of VIP on NO production is mediated primarily through VPAC1 and cAMP, we determined the effect of the VPAC1 antagonist and of the PKA inhibitor H89 on the changes induced by VIP in  $\kappa\text{B}$  and IRF-1 binding complexes. The inhibitory activity of VIP on LPS/IFN- $\gamma$ -mediated NF- $\kappa\text{B}$  binding was completely reversed by the VPAC1 antagonist (Fig. 9A, second panel, lane 3), but not by H89 (Fig. 9A, second panel, lane 4). However, both the VPAC1 antagonist and H89 reversed the VIP inhibition of IRF-1 binding (Fig. 9A, first panel, lanes 3 and 4). These results suggest that the inhibition of NF- $\kappa\text{B}$  and IRF-1 binding by VIP are mediated through VPAC1, but only the inhibition of the IRF-1 binding complex is entirely cAMP-dependent. This is supported by the fact that forskolin (a cAMP inducer) does not affect NF- $\kappa\text{B}$  binding (Fig. 9A, second panel, lane 5) but inhibits IRF-1 binding similar to VIP (Fig. 9A, first panel, lane 5 compared with lane 1).

To determine whether VIP reduces the levels of nuclear NF- $\kappa\text{B}$  and IRF-1 proteins, we performed Western blots (Fig. 9B). High levels of p50, p65, and IRF-1 were present in nuclear extracts obtained from Raw 264.7 cells stimulated with LPS/IFN- $\gamma$  (Fig. 9B, lane 1). In cells treated with VIP, the p50 levels remained constant, but both p65 and IRF-1 became undetectable (Fig. 9B, lane 2). The VPAC1 antagonist restored both p65 and IRF-1 levels

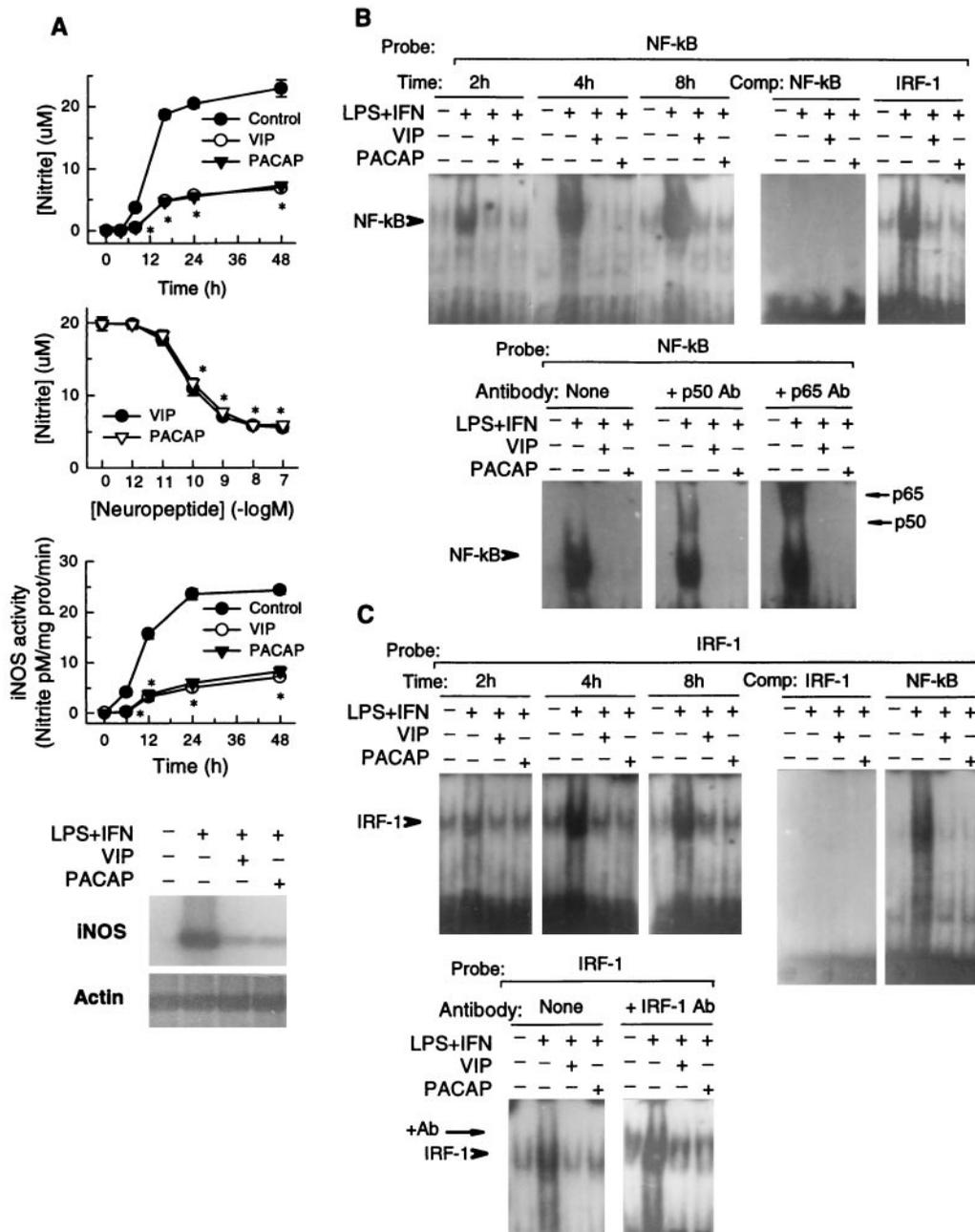


**FIGURE 7.** VIP and PACAP inhibit iNOS mRNA expression. Macrophages were stimulated with LPS (500 ng/ml) and IFN- $\gamma$  (200 U/ml), and incubated with or without VIP or PACAP ( $10^{-8}$  M) for 4, 8, 16, and 24 h. Cells incubated with medium alone were used as basal iNOS mRNA level controls (lane 1, for each time point). Expression of iNOS and  $\beta$ -actin mRNA was analyzed by Northern blot analysis at the indicated time points. Results are expressed in arbitrary densitometric units normalized per the expression of  $\beta$ -actin in each sample. The dotted line represents normalized iNOS mRNA levels of unstimulated cells. One representative experiment of three is shown.

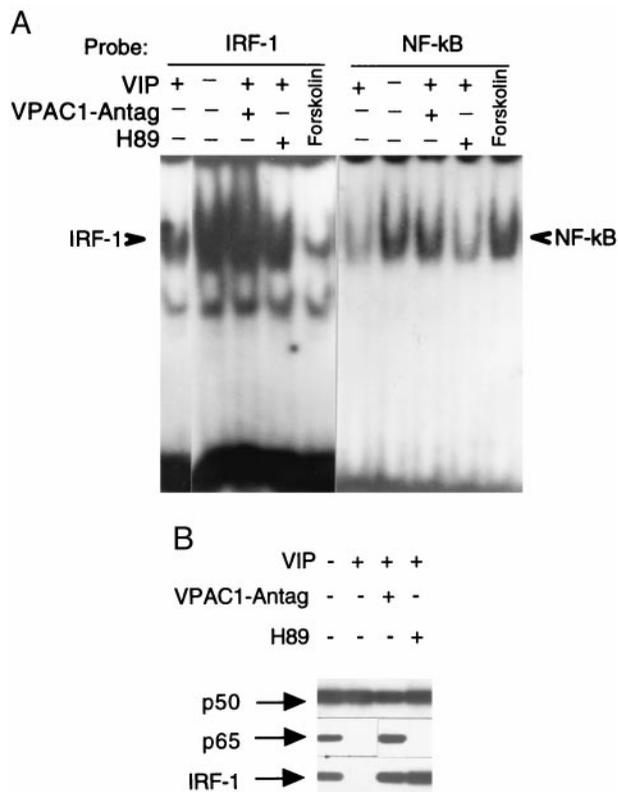
(Fig. 9B, lane 3), whereas the PKA inhibitor H89 restored only the IRF-1 levels (Fig. 9B, lane 4). These results indicate that the reductions in the levels of p65 and IRF-1 nuclear proteins are mediated through the VPAC1 receptor and confirm the involvement of cAMP in the inhibitory effect of VIP on IRF-1, but not on NF- $\kappa\text{B}$ .

#### VIP and PACAP inhibit NO production and iNOS expression in endotoxic mice

TNF- $\alpha$ , IL-1, IL-6, IFN- $\gamma$ , IL-12, and more recently NO have been shown to play pivotal roles in LPS-induced endotoxic shock (5, 6, 41–43). Indeed, inhibition of the LPS-induced cascade of proinflammatory cytokines is the primary mechanism through which anti-inflammatory immunomodulators such as IL-10, IL-11, and IL-13 confer protection against the lethal effects of LPS administration (44–48). Based on the ability of VIP and PACAP to downregulate cytokine production by LPS-activated macrophages, we reasoned that the *in vivo* protective effect of VIP/PACAP on endotoxin-induced lethal septic shock (17) might be mediated by a similar mechanism. To evaluate the effects of VIP and PACAP administration on NO production during lethal endotoxemia, mice were injected i.p. with a LD<sub>90</sub> of LPS (100  $\mu\text{g}$ ) concurrently with either medium or different amounts of VIP or PACAP (0.5–10 nmol). Serum and extracellular fluid (peritoneal lavage) were collected at different time points, and NO production was approximated from nitrite levels. Serum and peritoneal nitrite levels peaked 4 h following LPS administration, and levels were still relatively high at 8 h (Fig. 10A). In contrast, mice receiving VIP or PACAP in combination with LPS showed a significant reduction in serum and peritoneal NO levels (Fig. 10A). The inhibitory effect was dose-dependent, with a maximum for 5–10 nmol VIP/PACAP



**FIGURE 8.** VIP and PACAP inhibit the binding of NF- $\kappa$ B and IRF-1 to the iNOS promoter. *A*, VIP and PACAP inhibit NO production, iNOS activity and iNOS mRNA expression in LPS/IFN- $\gamma$ -stimulated Raw 264.7 macrophages. Raw 264.7 cells were stimulated with LPS (500 ng/ml) and IFN- $\gamma$  (200 U/ml) in the absence or presence of  $10^{-8}$  M VIP or PACAP (*first, third, and fourth panels*), or of a concentration range of either VIP or PACAP (*second panel*). Supernatants collected at different times (*first and third panels*), or 24 h (*second panel*) were assayed for nitrite production (*first and second panels*) and iNOS activity (*third panel*). Control cultures were incubated with LPS/IFN- $\gamma$  alone. Each result is the mean  $\pm$  SD of five separate experiments performed in duplicate. \*,  $p < 0.001$  with respect to control cultures with LPS alone. *Fourth panel*, Expression of iNOS mRNA expression was analyzed by Northern blot analysis at 12 h after LPS stimulation. Cells incubated with medium alone were used as basal iNOS mRNA level controls (*lane 1*). One representative experiment of three is shown. *B*, VIP and PACAP inhibit NF- $\kappa$ B binding to the iNOS promoter. *Upper panel*, Nuclear extracts were prepared from Raw 264.7 cells incubated for 2, 4, and 8 h with LPS (500 ng/ml) and IFN- $\gamma$  (200 U/ml) in the presence or absence of VIP or PACAP ( $10^{-8}$  M). NF- $\kappa$ B binding was assessed by EMSA using a radiolabeled oligonucleotide containing the murine NF- $\kappa$ B site of the iNOS promoter. Specificity was conducted by the addition of 50-fold excess of unlabeled homologous (NF- $\kappa$ B) or nonhomologous (IRF-1) oligonucleotides to nuclear extracts (Comp). *Lower panel*, Identification of the proteins bound to the NF- $\kappa$ B site using supershift analysis. Nuclear extracts (8 h incubation) were incubated with polyclonal Abs against p65 or p50 for 20 min before adding the probe. Similar results were observed in three independent experiments. *C*, VIP and PACAP inhibit IRF-1 binding to the iNOS promoter. *Upper panel*, Nuclear extracts were prepared from Raw 264.7 cells incubated for 2, 4, and 8 h with LPS (500 ng/ml) and IFN- $\gamma$  (200 U/ml) in the presence or absence of VIP or PACAP ( $10^{-8}$  M). IRF-1 binding was assessed by EMSA using a radiolabeled oligonucleotide containing the murine IRF-1 site of the iNOS promoter. Specificity was conducted by the addition of 50-fold excess of unlabeled homologous (IRF-1) or nonhomologous (NF- $\kappa$ B) oligonucleotides to nuclear extracts (Comp). *Lower panel*, Identification of the proteins bound to the IRF-1 site using supershift analysis. Nuclear extracts (8 h incubation) were incubated with polyclonal Abs against IRF-1 for 20 min before adding the probe. Arrow indicates the supershifted IRF-1-specific band. Similar results were observed in three independent experiments.



**FIGURE 9.** Specific receptors and intracellular pathways involved in the VIP and PACAP regulation of nuclear factors. *A*, Nuclear extracts prepared from LPS/IFN- $\gamma$ -stimulated Raw 264.7 cells were incubated with the NF- $\kappa$ B or IRF-1 oligonucleotides and subjected to EMSA. *Lane 1*, LPS + IFN- $\gamma$  + VIP; *lane 2*, LPS + IFN- $\gamma$ ; *lane 3*, LPS + IFN- $\gamma$  + VIP + VPAC1 antagonist; *lane 4*, LPS + IFN- $\gamma$  + VIP + H89; *lane 5*, LPS + IFN- $\gamma$  + forskolin. One representative experiment of four is shown. *B*, Nuclear extracts prepared from LPS/IFN- $\gamma$ -stimulated Raw 264.7 cells were subjected to Western blot analysis for p50, p65, and IRF-1. *Lane 1*, LPS + IFN- $\gamma$ ; *lane 2*, LPS + IFN- $\gamma$  + VIP; *lane 3*, LPS + IFN- $\gamma$  + VIP + VPAC1 antagonist; *lane 4*, LPS + IFN- $\gamma$  + VIP + H89. One representative experiment of three is shown.

(Fig. 10A, lower panels). Consistent with previous findings (49), serum and peritoneal TNF- $\alpha$ , IL-6, IL-12, and IFN- $\gamma$  levels were significantly reduced by VIP and PACAP in these samples (not shown). In addition, VIP and PACAP significantly inhibited iNOS activity and transcription in peritoneal cells in endotoxemic mice 2, 4, and 8 h after LPS challenge (Fig. 10, B and C).

## Discussion

Despite its beneficial role in host defense, excessive NO production has been implicated in the pathogenesis of several types of tissue injury, including ischemia-reperfusion injury of myocardium and intestine, oxidant lung injury, *N*-methyl-D-aspartate glutamate neurotoxicity, neuronal death after cerebral ischemia, endotoxemia and endotoxic shock, and cytokine-induced hypotension (reviewed in Ref. 8). Because VIP appears to prevent some of the types of tissue injury that NO promotes (50), we asked whether VIP and PACAP could interfere with the production of NO, especially during injury and inflammation. Because NO synthesis may result from either the constitutive nNOS and eNOS (neuronal and endothelial) or iNOS (macrophage) activity, the effects of VIP/PACAP may be different depending on the type of NOS. Indeed, previous reports indicated that VIP inhibits NO toxicity, but not synthesis in an acute lung injury model, which is dependent on nNOS (8, 18, 19). Also, a recent report indi-

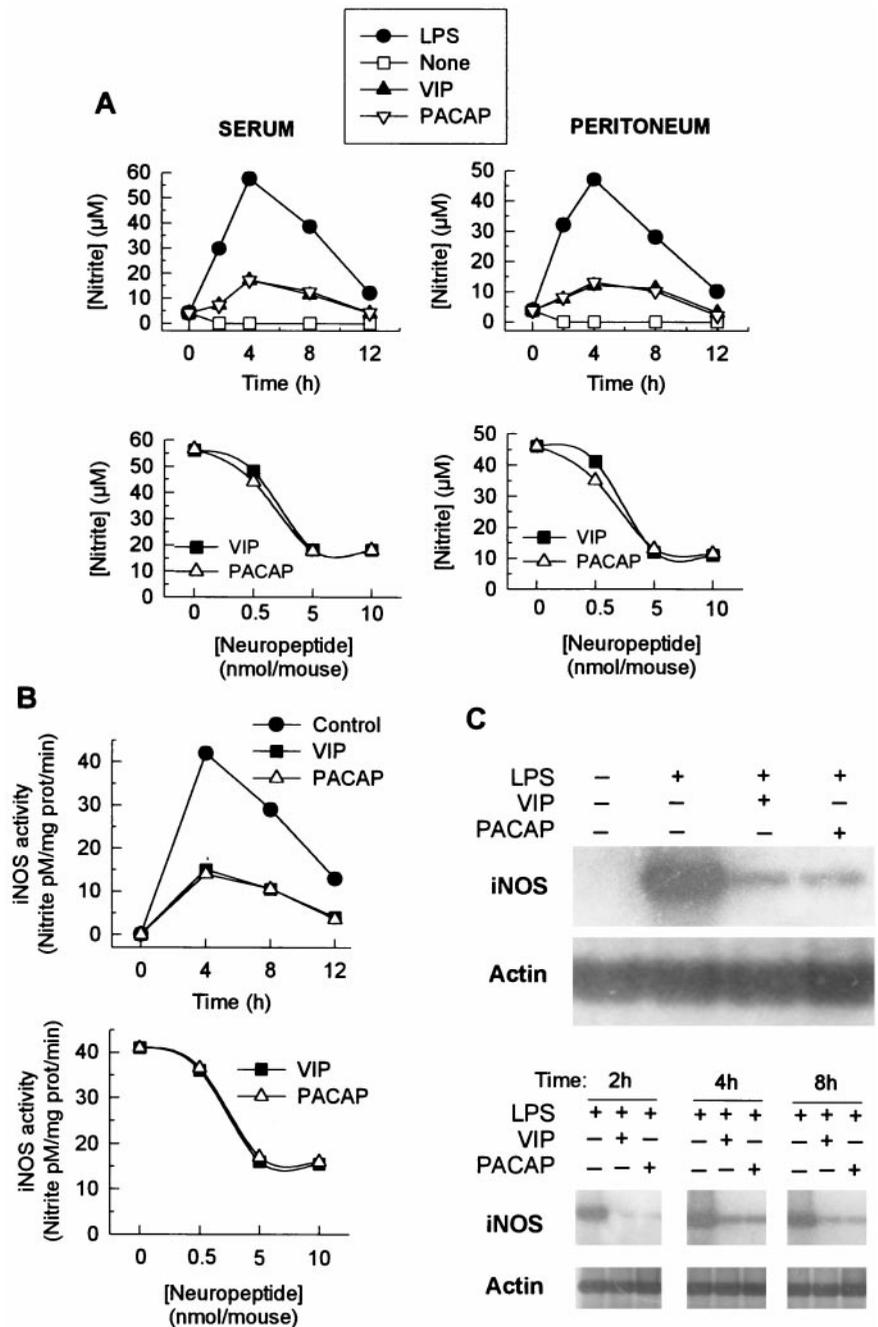
cates that VIP inhibits iNOS expression in stomach, spleen, and rectum, whereas augmenting nNOS in brain (51). In the present study, we investigated the effects of VIP and the structurally related neuropeptide PACAP on NO production and on the expression of iNOS (protein and mRNA) in murine macrophages activated with different stimuli. Both neuropeptides inhibit, in a similar way, NO production by peritoneal macrophages stimulated with LPS and/or IFN- $\gamma$ . The inhibitory effect is dose-dependent within a wide range of neuropeptide concentrations ( $10^{-7}$  to  $10^{-11}$  M), with the maximum effect being observed at  $10^{-8}$  M. This is the dose range at which VIP and PACAP modulate several immunological functions (11, 12).

Similar to the effect on cytokines such as IL-2, IL-6, IL-10, IL-12, and TNF- $\alpha$  (13, 16, 52, 53; Footnote 4), the inhibition of NO production requires intact VIP/PACAP molecules. This is in agreement with previous reports showing that both C- and N-terminal truncations of VIP lead to significant losses in biological activity (54, 55). Peritoneal macrophages and the Raw 264.7 macrophage cell line have been shown to constitutively express VPAC1 and PAC1 mRNA and VPAC2 mRNA following LPS-stimulation (31–33; Footnote 4). Our agonist studies suggest that VPAC1 is the major mediator of the VIP/PACAP inhibitory effect on NO generation. The VPAC2 agonist Ro 25-1553 was much less efficient; however, because VPAC2 is expressed only later in activated macrophages, the lack of effectiveness for the Ro compound may be due to a lack of appropriate receptors during the early culture period. The role of VPAC1 as the major player in mediating the effect of VIP/PACAP on NO production is also supported by the fact that a VPAC1 antagonist, but not PACAP<sub>6–38</sub>, an antagonist specific for PAC1 and to a lesser degree for VPAC2 (37), reverses the inhibitory effect of VIP/PACAP. Also, the VPAC1 antagonist blocked the effect of VIP/PACAP on IRF-1 and NF $\kappa$ B binding to the iNOS promoter, supporting the involvement of the VPAC1 in the inhibition of iNOS expression.

The VPAC1 is coupled primarily to the adenylate cyclase system (56), and NO production is indeed inhibited by agents that increase intracellular cAMP levels (39). In the present study, VIP and PACAP induced intracellular cAMP in a dose-dependent manner, and forskolin and PGE<sub>2</sub>, two strict cAMP-inducing agents, inhibited NO generation. In addition, H89, a potent and selective PKA inhibitor, reversed the inhibitory effect of VIP/PACAP, supporting the involvement of the cAMP/PKA pathway. However, because reversal was incomplete, a second cAMP-independent pathway may participate in the transduction of the VIP/PACAP signal. Similar observations were previously made for the inhibitory effect of VIP/PACAP on TNF- $\alpha$  and IL-12 production in macrophages (33; Footnote 4) and on IL-2 and IL-10 production in lymphocytes (our unpublished observations). The existence of a second cAMP-independent pathway is also supported by the fact that at concentrations that are physiologically relevant for VIP ( $10^{-9}$  M), the peptide induced less cAMP than forskolin, while acting as a more potent NO inhibitor. The nature of this second transduction pathway remains to be determined.

The iNOS activity is regulated at various levels, such as transcriptional, posttranscriptional, translational, and posttranslational. However, iNOS transcription appears to be the primary regulatory site (57). The present study indicates that the inhibitory effect of VIP and PACAP on NO production occurs through the reduction in iNOS protein and mRNA levels. The precise molecular mechanisms that account for the VIP/PACAP inhibition of iNOS expression are largely unknown, and it remains to be established whether the reduction in steady-state iNOS mRNA levels results from a decrease in de novo transcriptional rate, message stabilization, or both. However, some evidence points to a direct effect of

**FIGURE 10.** In vivo effects of VIP and PACAP on endotoxin-induced NO production. **A**, Time course and dose-response curve for the in vivo inhibitory effect of VIP/PACAP on NO production. Mice (groups of 3) were injected i.p. with LPS (100  $\mu\text{g}/\text{mouse}$ ) in the absence or presence of VIP or PACAP (5 nmol/mouse) (*upper panels*) or of different concentrations of VIP or PACAP (0.5–10 nmol/mouse) (*lower panels*). Serum and peritoneal exudate fluid were obtained at the indicated time points (*upper panels*) or at 4 h (*lower panels*) as described in *Materials and Methods* and were assayed for nitrite production. Each result is the mean  $\pm$  SD of three experiments performed in duplicate. **B**, VIP and PACAP inhibit in vivo iNOS activity in endotoxin-stimulated macrophages. Mice were i.p. injected with LPS (100  $\mu\text{g}/\text{mouse}$ ) in the absence or presence of VIP or PACAP (5 nmol/mouse) (*upper panels*) or of different concentrations of VIP or PACAP (0.5–10 nmol/mouse) (*lower panels*). Peritoneal macrophages were obtained at the indicated time points (*upper panels*) or at 4 h (*lower panels*) as described in *Materials and Methods* and were assayed for iNOS activity. Each result is the mean  $\pm$  SD of three experiments performed in duplicate. **C**, VIP and PACAP inhibit iNOS transcription in peritoneal macrophages from endotoxic mice. Expression of iNOS mRNA in peritoneal exudate cells was analyzed by Northern blot analysis at different time periods (2, 4, and 8 h) after LPS stimulation. Mice injected with medium alone were used as basal iNOS mRNA level controls (*upper panel, lane 1*, 4 h incubation). One representative experiment of three is shown.



VIP/PACAP on the de novo transcription as the most likely possibility. Delayed treatment with VIP/PACAP did not affect NO production, suggesting that VIP and PACAP inhibit an early event in iNOS expression. The promoter of the iNOS gene contains two major discrete regions that synergize upon binding of transcriptional factors (28, 29, 40, 58, 59): a NF- $\kappa$ B binding site, activated mainly by LPS, and an IRF-1 binding site, for IFN- $\gamma$ -induced transcriptional factors. As VIP and PACAP inhibit NO production induced by LPS and IFN- $\gamma$ , the two neuropeptides might regulate the activation of both transactivating sites. The present study indicates that VIP and PACAP inhibit NF- $\kappa$ B (p50/p65) binding and nuclear p65 protein levels in LPS/IFN- $\gamma$ -stimulated Raw 264.7 cells. We have recently reported a similar effect for the  $\kappa$ B site specific for the TNF- $\alpha$  promoter (33). It remains to be determined whether the VIP/PACAP reduction in NF- $\kappa$ B binding and nuclear p65 levels are due to an increase in I $\kappa$ B protein levels, a decrease

in I $\kappa$ B degradation, or/and inhibition in I $\kappa$ B phosphorylation, as described for other anti-inflammatory agents, such as IL-11, IL-10, TGF- $\beta$ 1, glucocorticoids, and antioxidants (44, 60–63).

In addition, treatment of the Raw 264.7 cells with LPS plus IFN- $\gamma$  resulted in a marked IRF-1 binding that was strongly inhibited by VIP or PACAP. Similar to p65, the levels of nuclear IRF-1 protein were reduced by VIP. Although the precise mechanism underlying this effect remains to be determined, this finding points to a VIP/PACAP-sensitive step in the activation of IRF-1, which unlike NF- $\kappa$ B is not constitutively present in the cytosol in an inactive form, but synthesized de novo following exposure to IFN- $\gamma$  (64). In macrophages, the IRF-1 gene responds to IFN- $\gamma$ , through binding of the IFN-gamma activation factor complex generated by the Jak1/2-STAT1 pathway (reviewed in Ref. 65). Could VIP and PACAP affect the Jak-STAT signaling? There is indeed evidence that cAMP-elevating agents inhibit the activation of the

STAT1 pathway (66–68). We investigated the relationship between the cAMP/PKA pathway and the IRF-1 binding activity. Both VIP and forskolin inhibit IRF-1 binding, and H89 reverses the effect of VIP on IRF-1 binding. The inhibition of IRF-1 binding could be due to a reduction in IRF-1 expression, most probably by down-regulating STAT1 activation. In contrast to IRF-1, increases in cAMP do not appear to directly affect NF- $\kappa$ B binding. Forskolin does not affect NF- $\kappa$ B binding, and H89 does not reverse the inhibitory effect of VIP. Similar results were obtained for the NF- $\kappa$ B binding to the TNF- $\alpha$  promoter in LPS-stimulated macrophages (33). We propose that, similar to TNF- $\alpha$  transcription, the  $\kappa$ B transactivation event required for iNOS expression is mediated through the cAMP-independent transduction pathway.

Production of NO by iNOS is beneficial for the protection against bacteria, fungi, parasites, viruses, and tumor cells (1, 4), but its overproduction can be harmful in endotoxemia, neurologic disorders, rheumatoid arthritis, and autoimmune diseases (5–7). Inhibitors of all NOS isoforms, which have been used to treat some of these diseases, led to significant harmful side-effects, such as hepatic injury, and even increased mortality (69, 70). Also, the clinical use of the NOS inhibitors has been hampered by the hypertensive effect resulting from the nonspecific inhibition of the constitutive NOS isoforms. Therefore, a specific iNOS inhibitor, with little or no effect on nNOS, holds significant therapeutical potential. The iNOS specificity of VIP initially documented by Bandyopadhyay et al. in rat (51) has to be confirmed in other species, and the molecular mechanisms allowing the differential regulation of iNOS and nNOS have to be clarified. However, based on their ability to down-regulate iNOS expression and NO production in endotoxemic mice, as described in this study, VIP/PACAP are attractive candidates for the development of treatments for septic shock and other acute inflammatory diseases, as well as for autoimmune diseases. In fact, VIP and PACAP protect mice from endotoxic shock even when administered post-LPS (17). Unlike neutralizing Abs and receptor antagonists directed against a single cytokine, VIP and PACAP reduce the production of a wide spectrum of proinflammatory mediators. In vitro and in vivo studies indicate that VIP and PACAP directly interact with macrophages to suppress the LPS-induced production of TNF- $\alpha$ , IL-6, IFN- $\gamma$ , IL-12, and NO (13, 16, 33).

In conclusion, we have shown that the binding of VIP and PACAP, primarily to VPAC1, inhibits iNOS expression at a transcriptional level in LPS/IFN- $\gamma$ -stimulated macrophages through two intracellular pathways: a cAMP-dependent pathway that preferentially inhibits IRF-1 transactivation and a cAMP-independent pathway that blocks NF- $\kappa$ B binding to the iNOS promoter. The inhibition of iNOS transcription by VIP/PACAP may have therapeutical potential, because excessive NO production has been implicated in the tissue injuries characteristic for several inflammatory and autoimmune diseases.

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