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# Vasoactive Intestinal Peptide and Pituitary Adenylate Cyclase-activating Polypeptide Inhibit Tumor Necrosis Factor α Transcriptional Activation by Regulating Nuclear Factor-kB and cAMP Response Element-binding Protein/c-Jun*

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**Tumor necrosis factor α (TNFα), an early cytokine produced by activated macrophages, plays an essential role in normal and pathological inflammatory reactions. The excessive production of TNFα is prevented by the so-called "macrophage-deactivating factors." This study examines the role of two structurally related neuropeptides, the vasoactive intestinal peptide (VIP) and the pituitary adenylate cyclase-activating peptide (PACAP), as inhibitors of TNFα. Both VIP and PACAP inhibit TNFα production from lipopolysaccharide-stimulated RAW 246.7 cells in a dose- and time-dependent manner. Although the activated cells express mRNA for all three VIP/PACAP receptors, agonist and antagonist studies indicate that the major receptor involved is $VIP_1R$. VIP/PACAP inhibit TNFα gene expression by affecting both NF-kB binding and the composition of the cAMP responsive element binding complex (CREB/c-Jun). Two transduction pathways, a cAMP-dependent and a cAMP-independent pathway, are involved in the inhibition of TNFα gene expression and appear to differentially regulate the transcriptional factors involved. Because TNFα plays a central role in various inflammatory diseases such as endotoxic shock, multiple sclerosis, cerebral malaria, and various autoimmune conditions, the down-regulatory effect of VIP/PACAP may have a significant therapeutic potential.**

Macrophages are widely recognized as cells that play a central role in the regulation of immune and inflammatory activities, as well as tissue remodeling. The execution of these activities is mediated by complex and multifactorial processes involving macrophage products (1). In response to antigens such as LPS,[1] macrophages secrete proinflammatory cytokines and oxidants such as TNFα, IL-6, IL-1β, IL-12, and nitric oxide (1). TNFα and IL-6 are important macrophage secretory products that contribute to pathophysiological changes associated with several acute and chronic inflammatory conditions, including septic shock, autoimmune diseases, wasting, rheumatoid arthritis, inflammatory bowel disease, and respiratory distress syndrome (2–4). A number of regulatory molecules termed macrophage-deactivating factors have been the focus of considerable research (5–9). These molecules are believed to prevent the excessive production of proinflammatory mediators, including TNFα and IL-6.

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 inhibit several macrophage functions, including phagocytosis, respiratory burst, and chemotaxis (reviewed in Ref. 10), as well as LPS-induced IL-6 production (11). Furthermore, we have recently demonstrated that VIP and PACAP protect mice from endotoxic shock presumably through the inhibition of TNFα and IL-6 production.[2] VIP was also reported to suppress TNFα production in human peripheral blood cells (12, 13).

Both VIP and PACAP interact with a family of three VIP/PACAP receptors, $VIP_1R$, and $VIP_2R$, which exhibit similar affinities for the two neuropeptides and activate primarily the adenylate cyclase system, and PACAP-R, which exhibits a 2–3 orders of magnitude higher affinity for PACAP than for VIP and activates both the adenylate cyclase and phospholipase C systems (reviewed in Ref. 14). Peritoneal macrophages have been described to possess $VIP_1R$ and PACAP-R (15–17).

LPS is a major stimulus for the production of proinflammatory cytokines, including TNFα, from macrophages (1). TNFα synthesis is controlled at several levels. Whereas post-transcriptional, translational, and post-translational mechanisms play important roles, TNFα transcription appears to be the primary regulatory site. Although the TNFα promoter contains a complex array of transactivating binding sites, the kB and CRE elements appear essential for maximal TNFα

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[1] The abbreviations used are: LPS, lipopolysaccharide; TNFα, tumor necrosis factor α; IL, interleukin; VIP, vasoactive intestinal peptide; PACAP, pituitary adenylate cyclase-activating polypeptide; CRE, cAMP responsive element; CREB, CRE-binding protein; CBP, CREB-binding protein; $PGE_2$, prostaglandin $E_2$; NF-kB, nuclear factor kB; ELISA, enzyme-linked immunosorbent assay; Ab, antibody; PCR, polymerase chain reaction; bp, base pair(s); H89, *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-iso-quinolinesulfonamide; EMSA, electrophoretic mobility shift assay; FK, forskolin.

[2] Delgado, M., Martinez, C., Pozo, D., Leceta, J., Calvo, J. R., Ganea, D., and Gomariz, R. P. (1998) *J. Immunol.*, in press.

transcription (18–21).

To further understand the molecular mechanism through which VIP and PACAP attenuate the inflammatory response, we have examined the effects of both neuropeptides on TNFα protein and mRNA levels in LPS-activated Raw 264.7 macrophages and sought the specific receptor, the intracellular signal pathway, as well as the possible nuclear factors involved.

## EXPERIMENTAL PROCEDURES

*Reagents*—Synthetic VIP, PACAP38, $VIP_{1-12}$, and $VIP_{10-28}$ were purchased from Novabiochem (Laufelfingen, Switzerland). The $VIP_1R$-antagonist (Ac-$His^1$,D-$Phe^2$,$Lys^{15}$,$Arg^{16}$,$Leu^{27}$)VIP(3-7)-GRF(8-27) and the $VIP_1R$-agonist ($Lys^{15}$,$Arg^{16}$,$Leu^{27}$)VIP(1-7)-GRF(8-27) were kindly donated by Dr. Patrick Robberecht (Universite Libre de Bruxelles, Belgium). The $VIP_2R$-agonists Ro 25-1392 Ac-($Glu^8$,O-$CH_3$-$Tyr^{10}$,$Lys^{12}$,$Nle^{17}$,$Ala^{19}$,$Asp^{25}$,$Leu^{26}$,$Lys^{27,28}$)-VIP cyclo(21-25) and Ro 25–1553 Ac-($Glu^8$,$Lys^{12}$,$Nle^{17}$,$Ala^{19}$,$Asp^{25}$,$Leu^{26}$,$Lys^{27,28}$,$Gly^{29,30}$,$Thr^{31}$)-VIP cyclo(21-25) were generous gifts from Drs. Ann Welton and David R. Bolin (Hoffmann-La Roche). The PACAP-R agonist maxadilan was a generous gift from Dr. Ethan A. Lerner (Massachusetts General Hospital, Charlestown, MA). The lipophilic VIP agonist stearyl-norleucine$^{17}$ VIP (SNV) and antagonist stearyl-norleucine$^{17}$ neurotensin-VIP hybrid (SANV) were previously described (22). The PACAP-R-antagonist $PACAP_{6-38}$, secretin, and glucagon were obtained from Peninsula Laboratories (Belmont, CA). LPS (from *E. coli* 055:B5), calphostin C, and forskolin were purchased from Sigma, and *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-iso-quinolinesulfonamide (H89) from ICN Pharmaceuticals, Inc. (Costa Mesa, CA). Antibodies against CREB, p50, p65, and c-Jun were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

*Cell Lines and Cell Culture*—The murine macrophage cell line Raw 264.7 (ATCC, Manassas, VA) was grown as recommended by ATCC. The cells were seeded in flat bottom 96-well microtiter plates (Corning Glass, Corning, NY) at $8 \times 10^4$ cells/well in a final volume of 200 μl. 24 h later the monolayers were washed twice in Dulbecco's modified Eagle's medium without serum, and 200 μl of 10% Dulbecco's modified Eagle's medium was added to each well. The cells were stimulated with LPS in the presence or absence of VIP or PACAP38. Cell-free supernatants were harvested at designated time points and assayed for TNFα production by ELISA.

*Cytokine Determination: ELISA Assay for TNFα*—The amount of soluble TNFα was determined by using a murine TNFα ELISA assay with capture Ab (clone MP6-XT22) and detection Ab (biotinylated clone MP6-XT3) with murine TNFα (Pharmingen, San Diego, CA) as standard. The ELISA is specific for murine TNFα (does not cross-react with human or rat TNFα, or with other murine cytokines such as IL-10, IL-6, IL-2, IL-3, IL-4, IL-5, IL-1α, IL-1β, and interferon-γ). The sensitivity of the assay is 10 pg TNFα/ml.

*RNA Isolation and Northern Blot Analysis*—Northern blot analysis was performed according to standard methods. The cells ($2 \times 10^6$ cells/ml) were stimulated with LPS (1 μg/ml) in the absence or presence of VIP or PACAP38 ($10^{-8}$ M) for different time periods, and total RNA was isolated with Ultraspec RNA reagent (Biotecx Labs, Houston, TX) as recommended by the manufacturer. 20 μg of total RNA were electrophoresed on 1.2% agarose-formaldehyde gels, transferred to S&S Nytran membranes (Schleicher & Schuell), and UV cross-linked. The TNFα probe oligonucleotide 5′-TTGACCTCAGCGCTGAGTTGGTC-CCCCTTCTCAGCTGGAAGACT-3′ was designed based on the published murine TNFα sequence (23). The following murine 18 S rRNA oligonucleotide 5′-CCAAGGACAGGGCCTCGAAAGAGTCCTGTA-3′ was used as control. The membranes were prehybridized for 16 h at 42 °C, followed by hybridization at 60 °C for 16 h with the appropriate probes. The prehybridization and hybridization buffers were purchased from 5 Prime → 3 Prime, Inc. (Boulder, CO).

*Reverse Transcription-PCR for the Detection of $VIP_1R$, $VIP_2R$, and PACAP-R mRNA*—2 μg of total RNA from unstimulated and LPS-stimulated Raw 264.7 cells was reverse transcribed and 3 μl of cDNA was amplified with specific primers. The primers for $VIP_2R$ and PACAP-R were designed based on published murine pancreatic $VIP_2R$ (24) and mouse brain PACAP-R (25), respectively. The primers for $VIP_1R$ were previously described (26, 27). The designated primers sequences are as follows: $VIP_1R$ sense, 5′-CCTTCTTCTCTGAGCGGAAG-TACTT-3′ and antisense, 5′-CCTGCACCTCACCATTGAGGAAGCAG-3′; $VIP_2R$ sense, 5′-GTCAAGGACAGCGTGCTCTACTCC-3′ and antisense, 5′-CCCTGGAAGGAACCAACACATAAG-3′; PACAP-R sense, 5′-CAAG AAGGAGCAAGCCATGTGC-3′ and antisense, 5′-CATCGAG-TAATGGGGGAAGGG-3′; β-actin sense, 5′-GATGGTGGGTATGGGT-CAGGGG-3′ and antisense, 5′-GCTCATTGCCGATAGTGATGACCT-3′. The expected sizes for the amplified fragments are: 450 bp for $VIP_1R$, 572 bp for $VIP_2R$, 317 bp for PACAP-R, and 660 bp for β-actin. The PCR conditions were: denaturation 94 °C, 45 s, annealing 55 °C, 45 s, primer extension 72 °C, 90 s for 35 cycles. The PCR products were size separated in 2% agarose gels.

*Preparation of Nuclear Extracts*—Nuclear extracts were prepared by the mini-extraction procedure of Schreiber *et al.* (28) with slight modifications. Raw 264.7 cells were plated at a density of $10^7$ cells/well in 6-well plates, stimulated and washed twice with ice-cold phosphate-buffered saline and 0.1% bovine serum albumin. 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 dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 5 mM NaF, 1 mM $Na_3VO_4$, and 1 mM $NaN_3$). After 15 min on ice, Nonidet P-40 was added to a final 0.5% concentration, and nuclei were isolated by centrifugation at 12,000 × *g* for 40 s. Pelleted nuclei were washed once with 0.2 ml of ice-cold buffer A and lysed by incubation for 30 min on ice in 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 dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 1 mM $NaN_3$). Supernatants containing nuclear proteins were harvested by centrifugation for 10 min at 14,000 rpm at 4 °C, the protein concentration was determined by the Bradford method, and aliquots were stored at −80 °C for use in electrophoretic mobility shift assays (EMSAs).

*EMSA*—Oligonucleotides corresponding to the proximal (−510 bp) kB3 (5′-CAAACAGGGGGCTTTCCCTCCTC-3′) and CRE (5′-TCCA-CATGAGATCATGGTTT-3′) (−106 bp) motif of the TNFα promoter (19, 23) were synthesized, annealed, and end-labeled with [γ-$^{32}$P]ATP. 20,000–50,000 cpm of double-stranded oligonucleotide, corresponding to approximately 0.5 ng, was used for each reaction. Binding reaction mixtures (15 μl) containing: 0.5–1 ng of DNA probe, 5 μg of nuclear extract, 2 μg of poly(dI-dC)·poly(dI-dC), and binding buffer (50 mM NaCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, 5% glycerol, and 10 mM Tris-HCl, pH 7.5) were incubated on ice for 15 min, followed by the addition of the probe. After 20 min of incubation at room temperature, the samples were loaded onto 4% nondenaturing polyacrylamide gel and electrophoresed in TGE buffer (50 mM Tris-HCl, pH 7.5, 0.38 M glycine, and 2 mM EDTA), followed by transfer and autoradiography. In competition and antibody supershift experiments, nuclear extracts were incubated for 15 min at room temperature with antibody (1 μg) or competing oligonucleotide (50-fold excess) before the addition of the labeled probe.

*JNK Activity Assay*—JNK activity was determined using a stress-activated protein kinase/JNK assay kit (New England BioLabs, Inc., Beverly, MA) according to the manufacturer's instructions. Briefly, Raw 264.7 cell lysates, prepared according to the manufacturer's instructions, were incubated with an N-terminal c-Jun (1–89) fusion protein bound to glutathione-Sepharose beads to selectively isolate JNK, followed by a kinase reaction in the presence of cold ATP. Proteins were resolved on 12% SDS-polyacrylamide gel electrophoresis gels, and electrophoretically transferred to nitrocellulose membranes. c-Jun phosphorylation at $Ser^{63}$ was selectively analyzed by immunoblotting using a phospho-specific c-Jun antibody and chemiluminescent detection.

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

## RESULTS

*VIP and PACAP Inhibit LPS-induced TNFα Production by Raw 264.7 Macrophages*—To investigate whether VIP/PACAP inhibit TNFα release, Raw 264.7 murine macrophages were stimulated with different concentrations of LPS in the absence or presence of various doses of VIP or PACAP, and the amount of TNFα released in the culture supernatants was assayed by ELISA at different time periods. VIP and PACAP inhibit the TNFα production by LPS-stimulated cells in a dose- and time-dependent manner, showing maximal effects at $10^{-8}$ M after 6 h of incubation (Fig. 1).

*The Inhibition of LPS-induced TNFα Production by VIP or PACAP Is Mediated through $VIP_1R$*—Next we investigated whether the inhibitory effects of VIP/PACAP could be related to

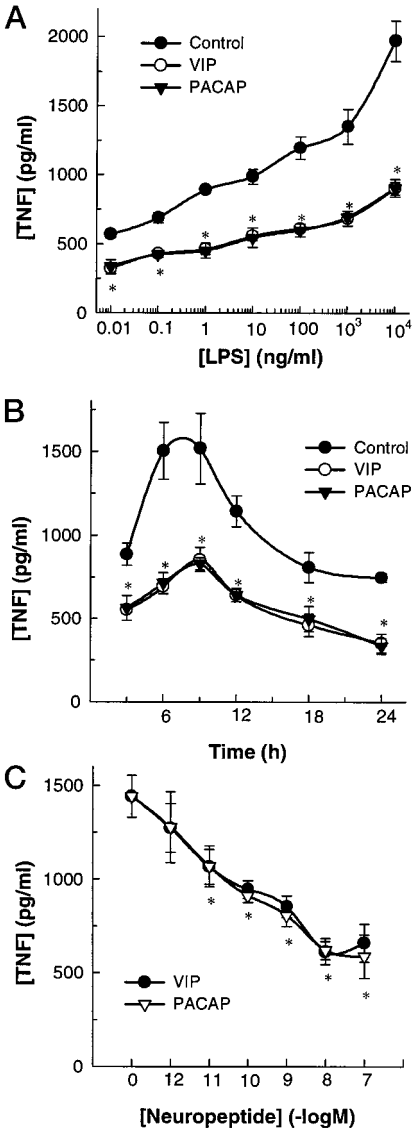


FIG. 1. **VIP and PACAP inhibit TNFα production by LPS-stimulated macrophages.** *A,* Raw 264.7 cells were stimulated with a concentration range of LPS (10 pg/ml to 10 μg/ml) in the absence or presence of $10^{-8}$ M VIP or PACAP. After a 6-h incubation period, supernatants were collected, and TNFα release was determined by ELISA. Control cultures were incubated with LPS alone. *B,* time course for the inhibitory effect of VIP/PACAP on TNFα production. Raw 264.7 cells were stimulated with LPS (1 μg/ml) in the absence or presence of $10^{-8}$ M VIP or PACAP. Supernatants collected at different times were assayed for TNFα production by ELISA. *C,* dose-response curve for the inhibitory effect of VIP and PACAP on TNFα production. Raw 264.7 cells were incubated with LPS (1 μg/ml) and a concentration range of either VIP or PACAP for 6 h. Supernatants were collected and TNFα release was determined by ELISA. For *A–C*, cells cultured in the absence of LPS with or without VIP/PACAP did not produce detectable levels of TNFα (<10 pg/ml). Each result is the mean ± S.D. of five separate experiments performed in duplicate. * $p < 0.001$ with respect to control cultures with LPS alone.

occupancy of specific receptors. First, we compared the effect of VIP/PACAP to secretin, glucagon, and the VIP- and PACAP-fragments $VIP_{1-12}$, $VIP_{10-28}$, and $PACAP_{6-38}$. TNFα production was inhibited by secretin only at $10^{-7}$ M, whereas no effect was observed with various concentrations of glucagon ($10^{-9}$-$10^{-7}$ M) (Fig. 2*A*). The two VIP fragments and $PACAP_{6-38}$ failed to inhibit TNFα production, suggesting that intact VIP and PACAP molecules are required for their inhibitory activity (Fig. 2*A*).

To determine which of the VIP/PACAP receptors were involved, we used specific receptor agonists and antagonists. We investigated the effect of a newly described $VIP_1R$-agonist (29), of two $VIP_2R$ agonists (Ro 25-1392 and Ro 25-1553) (30, 31), and of maxadilan, a specific PACAP-R agonist (32) on the LPS-induced TNFα production. $VIP_1R$, $VIP_2R$, and PACAP-R agonists inhibited TNFα release (Fig. 2*B*). The $VIP_1R$ agonist exhibited a similar potency as VIP/PACAP (60% inhibition), whereas maxadilan and the two Ro compounds were much less efficient (22–26% inhibition) (Fig. 2*B*). In addition, we investigated the ability of $PACAP_{6-38}$, an antagonist specific for PACAP-R and $VIP_2R$ (33), and of a specific $VIP_1R$-antagonist (34), to reverse the effects of VIP and PACAP. Increasing concentrations of the antagonists ($10^{-6}$-$10^{-8}$ 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 $VIP_1R$-antagonist in a dose-dependent manner (Fig. 2*C*). In contrast, $PACAP_{6-38}$ did not reverse the inhibitory effect of VIP or PACAP (Fig. 2*D*). Neither the $VIP_1R$-antagonist nor $PACAP_{6-38}$ significantly affected TNFα levels (Fig. 2, *C* and *D* legends). Furthermore, the simultaneous addition of VIP or PACAP and $VIP_1R$-agonist did not result in an additive effect on TNFα release (Fig. 2*E*). Together these results confirm the specificity of the VIP and PACAP inhibitory activity and suggest that both neuropeptides exert their action through binding to $VIP_1R$.

Finally, the simultaneous addition of VIP with maxadilan and/or Ro 25-1553 did not result in significant differences in comparison with samples treated with VIP alone; however, a small but statistically significant difference was observed when the cells were preincubated for 15 min with PACAP-R and/or $VIP_2R$ agonists before the addition of VIP (Fig. 2*F*).

*Raw 264.7 Cells Express $VIP_1R$, $VIP_2R$, and PACAP-R*—The fact that $VIP_1R$, $VIP_2R$, and PACAP-R agonists inhibit TNFα production suggests that Raw 264.7 cells express all three receptors. To test this possibility, we investigated the expression of $VIP_1R$, $VIP_2R$, and PACAP-R mRNA by reverse transcription-PCR in unstimulated and LPS-stimulated Raw 264.7 cells. Both $VIP_1R$- and PACAP-R-specific fragments were amplified from unstimulated and stimulated macrophages, whereas $VIP_2R$ fragments were only detected in stimulated cells (Fig. 3). These results indicate that LPS-stimulated Raw 264.7 cells express $VIP_1R$, $VIP_2R$, and PACAP-R mRNA.

*Intracellular Signal Pathways Involved in the Inhibitory Activity of VIP and PACAP on TNFα Production*—cAMP but not protein kinase C involvement. To study the second messengers involved in the inhibitory activity of VIP and PACAP, we investigated the effects of calphostin C (a protein kinase C inhibitor) (35) and of H89 (a protein kinase A inhibitor) (36) on the inhibition of TNFα. High concentrations (100 nM) of calphostin C inhibited TNFα production in LPS-treated cells (Fig. 4*C*); however, in the 1–10 nM concentration range, calphostin C did not affect TNFα production in LPS-stimulated cells and did not reverse the inhibitory effect of VIP or PACAP (Fig. 4, *A* and *B*). In contrast, H89 partially reversed the inhibitory effect of VIP and PACAP (Fig. 4, *A* and *B*). These results suggest that the inhibitory effect of VIP/PACAP is mediated, at least partially, through increases in intracellular cAMP.

*Involvement of a cAMP-independent Pathway*—The partial reversal of the inhibitory effect of VIP on TNFα production by H89 suggests the involvement of an additional cAMP-independent transduction pathway. To address this question, we used a lipophilic VIP agonist (SNV) and a lipophilic VIP antagonist (SANV) previously developed for the neurotrophic action of VIP, which act through cAMP-independent pathways (22). SNV inhibits TNFα production, although less efficiently than VIP or PACAP (Fig. 5*A*), and the inhibitory activity is not

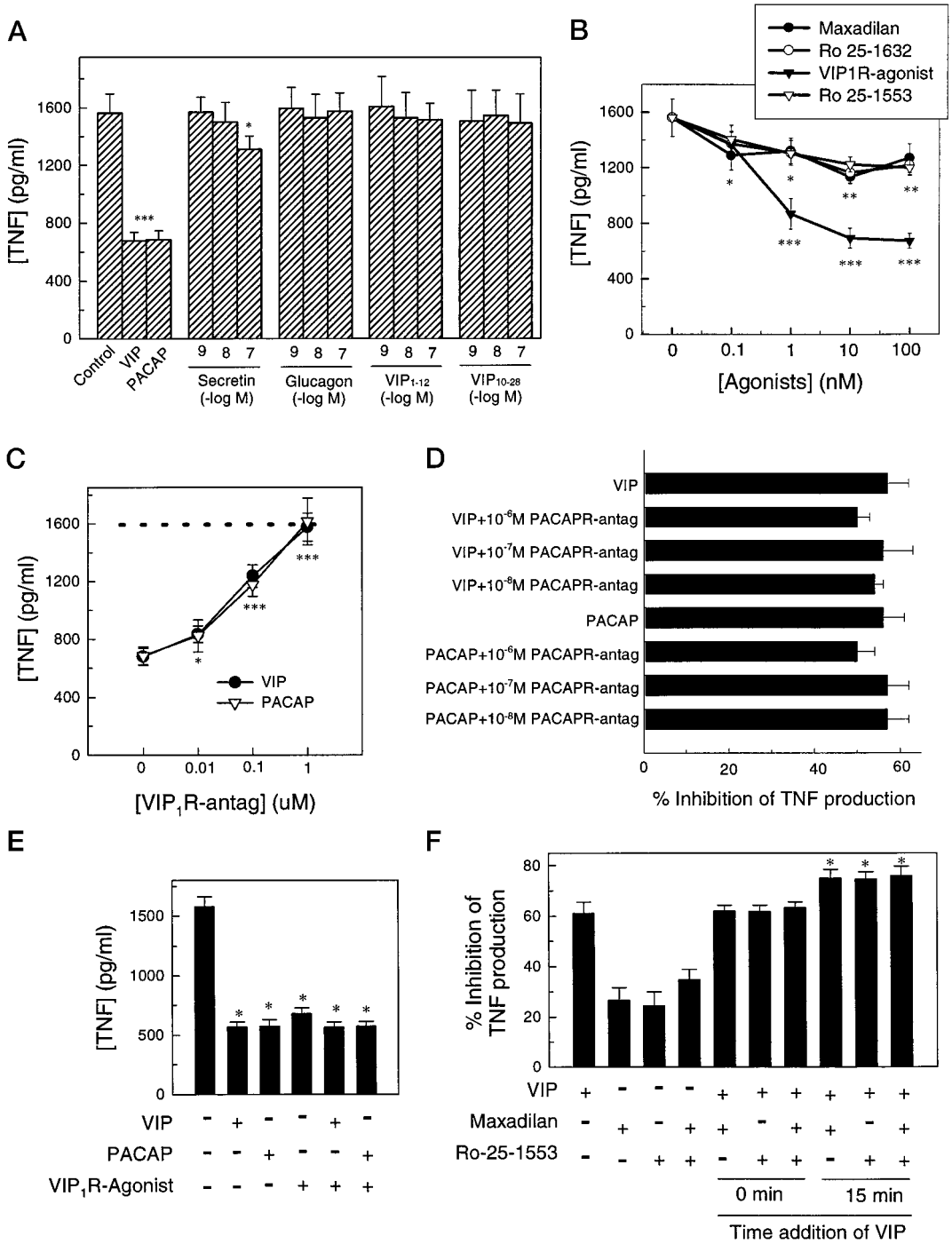


FIG. 2. **Inhibition of TNFα production by VIP and PACAP is specific and is mediated through $VIP_1R$.** *A* and *B,* comparative effects of VIP, PACAP38, VIP-related peptides, VIP fragments, and VIP and PACAP agonists on TNFα production. Raw 264.7 cells were stimulated with LPS (1 μg/ml) in the absence or presence of different concentrations of secretin, glucagon, $VIP_{1-12}$, and $VIP_{10-28}$ (*A*), or maxadilan (PACAP-R-agonist), Ro 25-1632, Ro 25-1553 ($VIP_2R$-agonists), or (Lys[15],Arg[16],Leu[27])VIP(1–7)-GRF(8–27) ($VIP_1R$-agonist) (*B*). Supernatants were collected 6 h later and assayed for TNFα production by ELISA. Each result is the mean ± S.D. of four experiments performed in duplicate. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ with respect to control cultures with LPS alone. *C* and *D,* effect of PACAP-R and $VIP_1R$ antagonists on the inhibitory activity of VIP and PACAP on TNFα production. Raw 264.7 cells were stimulated with LPS (1 μg/ml), and treated simultaneously with VIP or PACAP ($10^{-8}$ M), and different concentrations of the $VIP_1R$-antagonist (Ac-His[1],D-Phe[2],Lys[15],Arg[16],Leu[27])VIP(3–7)-GRF(8–27) (*C*), or the PACAP-R antagonist (PACAP6–38) (*D*). Supernatants were collected 6 h later and assayed for TNFα. $VIP_1R$ antagonist and PACAP6–38 did not affect TNFα levels (1577 ± 112 pg/ml for $10^{-6}$ M $VIP_1R$ antagonist; 1516 ± 145 pg/ml for $10^{-6}$ M PACAP6–38 compared with 1562 ± 135 pg/ml for LPS alone). Percentage of inhibition (*D*) was calculated by comparison with controls containing LPS alone. Each result is the mean ± S.D. of four experiments performed in duplicate. * $p < 0.05$, *** $p < 0.001$ compared with samples treated with neuropeptides and without antagonists. *E* and *F,* effect of $VIP_1R$, $VIP_2R$, and PACAP-R agonists. Raw 264.7 cells were stimulated with LPS (1 μg/ml) and treated with a $VIP_1R$ agonist (100 nM) (*E*), or maxadilan and/or Ro 25-1553 (100 nM) (*F*). VIP or PACAP ($10^{-8}$ M) were added at the same time or 15 min after the agonists (*F*). Supernatants were collected 6 h later and assayed for TNFα production. Percentage of inhibition (*F*) was calculated by comparison with controls containing LPS alone. Results are the mean ± S.D. of five experiments performed in duplicate. * $p < 0.001$ with respect to samples treated with VIP.

reversed by H89 (Fig. 5*B*). This suggests the existence of a second cAMP-independent pathway in the transduction of the VIP signaling in macrophages. To further substantiate this possibility, we investigated whether SNV contributes to the inhibition of TNFα by forskolin or prostaglandin $E_2$ ($PGE_2$), two strict cAMP-inducing agents. Although SNV did not significantly affect the VIP/PACAP inhibitory effect, it increased the inhibitory action of forskolin and $PGE_2$ (Fig. 5*C*). The effect is additive, and not synergistic, suggesting that the two pathways are probably not connected.

In addition, we investigated the effect of the antagonist SANV on the inhibition of TNFα production by VIP, PACAP, forskolin, $PGE_2$, and 8-Br-cAMP. SANV partially reverses the inhibitory effect of VIP, PACAP, forskolin, $PGE_2$, and 8-Br-cAMP (Fig. 5*D*), suggesting that SANV acts on a messenger downstream from cAMP.

*VIP and PACAP Regulate TNFα Production at a Transcriptional Level*—To determine whether the VIP/PACAP affect TNFα transcription, Raw 264.7 cells were stimulated with LPS in the presence or absence of $10^{-8}$ M VIP or PACAP for 1.5, 3, and 6 h, and total RNA was prepared and subjected to Northern blot analysis. Although no TNFα mRNA is detectable in unstimulated cells, time-dependent increasing levels of TNFα mRNA are present in LPS-stimulated cells (Fig. 6). At all three time points, VIP and PACAP significantly reduced the levels of specific TNFα mRNA (Fig. 6). These results indicate that both neuropeptides exert their action at a transcriptional level.

*VIP and PACAP Inhibit NF-kB Binding*—Activation and nuclear translocation of members of the NF-kB/c-Rel family constitutes the hallmark of macrophage stimulation by proinflammatory cytokines and bacterial products (37). To investigate whether VIP/PACAP affect NF-kB nuclear translocation, we used electrophoretic mobility shift assays. Stimulation of Raw 264.7 cells with LPS led to strong NF-kB binding compared with unstimulated cells, and treatment with VIP or PACAP significantly reduced the binding (Fig. 7*A*). The binding specificity was confirmed by using homologous (NF-kB) and nonhomologous (CRE) oligonucleotides as competitors (Fig. 7*B*). Furthermore, monospecific anti-p50 and anti-p65 Abs used in supershift experiments indicated that the LPS-induced kB binding complex was composed primarily of p50/p65 heterodimers (Fig. 7*C*).

*VIP and PACAP Modulate the Composition of the CRE Binding Complex*—Recently, it has been established that the CRE site in the TNFα promoter is required for optimal transcription of the TNFα gene in monocytes (20). The CRE binding activity is constitutively expressed in unstimulated Raw 264.7 cells, and treatment with LPS in the presence or absence of VIP/PACAP does not affect the binding (Fig. 8*A*). The specificity of the CRE binding activity was confirmed with homologous (CRE) or nonhomologous (NF-kB) oligonucleotides as competitors (Fig. 8*A*). Antibody supershift experiments were performed to determine the composition of the CRE binding complexes. In unstimulated cells, the majority of the complex was supershifted by an anti-CREB Ab, whereas no supershift was observed using an anti-c-Jun Ab (Fig. 8*B*). In contrast, a major supershift by the anti-c-Jun Ab was evident in cells treated with LPS (Fig. 8*B*), indicating the presence of c-Jun in the CRE binding complexes. Treatment of LPS-stimulated cells with VIP or PACAP led to complexes similar to those from unstimulated cells, containing CREB and minor amounts, if any, of c-Jun (Fig. 8*B*).

*VIP and PACAP Inhibit JNK Activity in LPS-stimulated Raw 264.7 Cells*—Phosphorylation of c-Jun at $Ser^{63}$ and $Ser^{73}$ by JNK after LPS stimulation is essential for the binding of the c-Jun protein to the CRE site (38–40). Because VIP and

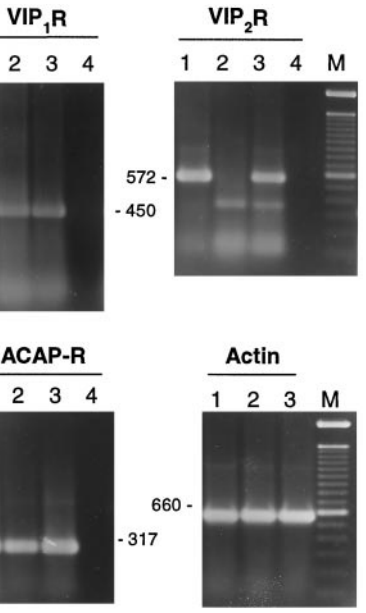


FIG. 3. **Expression of $VIP_1R$, $VIP_2R$, and PACAP-R mRNA in Raw 264.7 cells.** Total RNA extracted from unstimulated macrophages (*lane 2*) and LPS-stimulated macrophages (*lane 3*) ($2 \times 10^7$ cells) was subjected to reverse transcription-PCR with specific primers for $VIP_1R$, $VIP_2R$, PACAP-R, and β-actin as described under "Experimental Procedures." Brain RNA was used as a positive control (*lane 1*). Reactions without cDNA served as negative control (*lane 4*). Numbers indicate the predicted sizes for the amplified fragments. One representative experiment of two is shown.

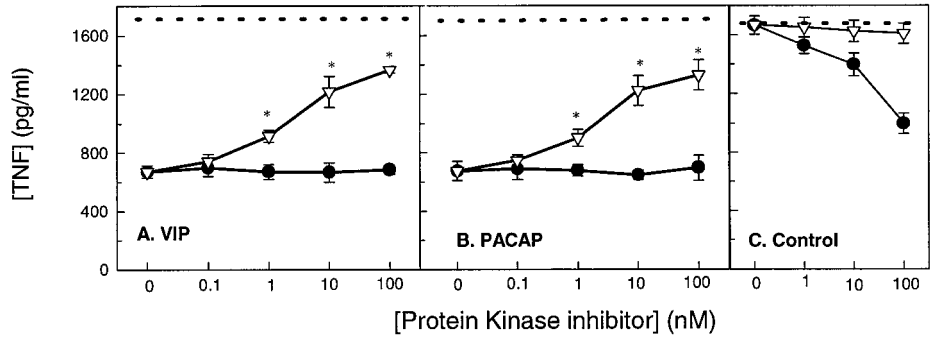


FIG. 4. **Comparative effects of calphostin C (a protein kinase C inhibitor) and H89 (protein kinase A inhibitor) on the inhibitory activity of VIP and PACAP.** Raw 264.7 cells were stimulated with LPS (1 μg/ml), LPS plus VIP ($10^{-8}$ M) (*A*), or LPS plus PACAP ($10^{-8}$ M) (*B*) in the absence or presence of different concentrations of calphostin C or H89. After a 6-h culture the supernatants were assayed for TNFα production. The *dashed horizontal line* represents control values from cultures incubated with LPS alone (1659 ± 141 pg TNFα/ml). Results are the mean ± S.D. of five experiments performed in duplicate. * $p < 0.001$ with respect to neuropeptide-treated samples without protein kinase modulators. ●, calphostin C; ▽, H89.

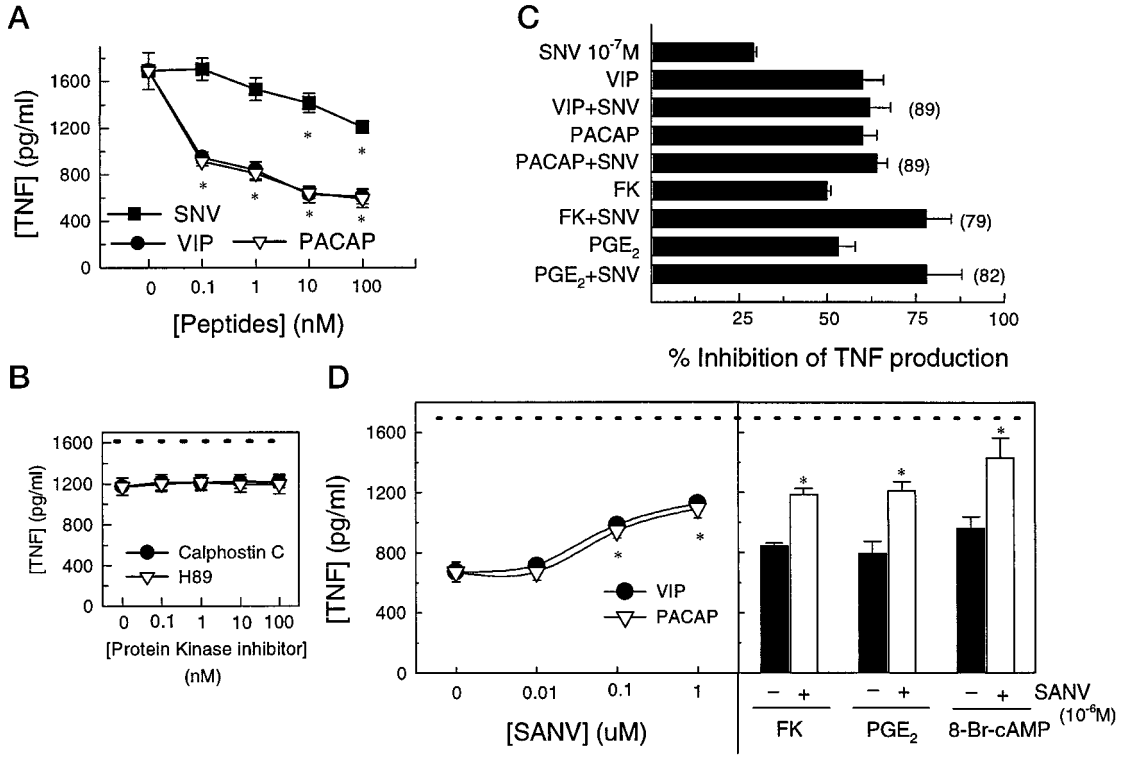


FIG. 5. **Involvement of a cAMP-independent pathway in the inhibitory effect of VIP/PACAP on TNFα production.** *A,* effect of the lipophilic VIP agonist SNV. Raw 264.7 cells were stimulated with LPS (1 μg/ml) and incubated with different concentrations of SNV, VIP, or PACAP. Supernatants were collected 6 h later and assayed for TNFα production. Each result is the mean ± S.D. of four experiments performed in duplicate. * $p < 0.001$ compared with controls with LPS alone. *B,* effect of calphostin C and H89 on the inhibitory activity of SNV. Raw 264.7 cells were stimulated with LPS (1 μg/ml) or LPS plus SNV ($10^{-7}$ M) in the absence or presence of different concentrations of calphostin C or H89. Supernatants were collected 6 h later and assayed for TNFα production. The *dashed horizontal line* represents control values from cultures with LPS alone. *C,* effect of SNV on the inhibitory action of VIP, PACAP, and other cAMP-elevating agents. Raw 264.7 cells were stimulated with LPS (1 μg/ml) and incubated with VIP ($10^{-8}$ M), PACAP ($10^{-8}$ M), FK ($10^{-6}$ M), or $PGE_2$ ($10^{-6}$ M) in the presence or absence of 100 nM SNV. Supernatants were collected 6 h later and assayed for TNFα production. Percentage of inhibition was calculated by comparison with LPS controls (1,690 ± 159 pg TNFα/ml). Numbers in parenthesis represent expected percentages of inhibition if the effects of SNV and others agents were additive. Each result is the mean ± S.D. of four experiments performed in duplicate. *D,* effect of SANV, a lipophilic VIP antagonist, on the inhibitory activity of VIP, FK, 8-Br-cAMP, and $PGE_2$. Raw 264.7 were stimulated with LPS (1 μg/ml) and incubated with VIP ($10^{-8}$ M), PACAP ($10^{-8}$ M), FK ($10^{-6}$ M), 8-Br-cAMP ($10^{-7}$ M), or $PGE_2$ ($10^{-6}$ M) in the presence or absence of different concentrations of SANV. Supernatants were collected 6 h later and assayed for TNFα production. The *dashed horizontal line* represents control values from cultures incubated with LPS alone. Results are the mean ± S.D. of five experiments performed in duplicate. * $p < 0.001$ with respect to samples treated with VIP.

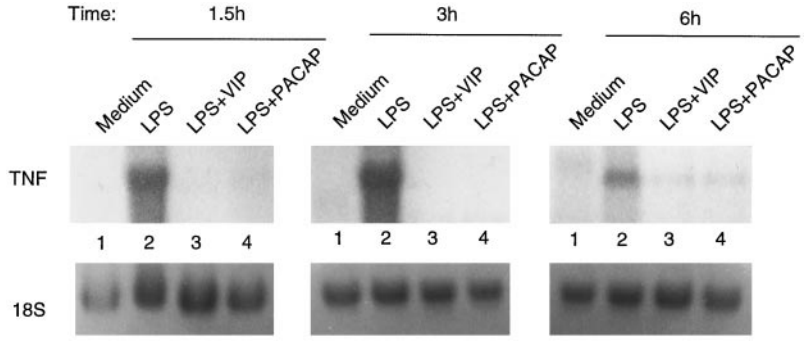


FIG. 6. **VIP and PACAP inhibit TNFα transcription.** Raw 264.7 cells ($2 \times 10^7$ cells) were stimulated with LPS (1 μg/ml) and incubated with or without VIP or PACAP ($10^{-8}$ M) for 1.5, 3, or 6 h. Cells incubated with medium alone were used as basal TNFα mRNA level controls. Total RNA was extracted and the expression of TNFα and 18 S mRNA was analyzed by Northern blot analysis. One representative experiment of three is shown.

PACAP reduce c-Jun in the CRE binding complexes, we investigated whether VIP inhibits JNK activity. Unstimulated and LPS-stimulated cells were incubated with or without VIP for 1–6 h, and the presence of phosphorylated c-Jun was determined by Western blot. LPS-stimulated cells express high levels of phosphorylated c-Jun in comparison with unstimulated cells (Fig. 9). VIP reduces the expression of c-Jun in a time-dependent manner, with the highest effect at 2 and 3 h after stimulation (Fig. 9).

*Involvement of $VIP_1R$ and cAMP in the Effects of VIP on kB and CRE Binding*—Because the inhibitory effect of VIP on TNFα production is mediated primarily through $VIP_1R$ and cAMP, we determined the effect of the $VIP_1R$ antagonist and of the protein kinase A inhibitor H89 on the changes induced by VIP in kB and CRE binding complexes. The inhibitory activity of VIP on LPS-mediated NF-kB binding was completely reversed by the $VIP_1R$-antagonist (Fig. 10*A*, *lane 4*), and only partially by H89 (Fig. 10*A*, *lane 3*). However, both the $VIP_1R$ antagonist and H89 reversed the changes in the composition of the CRE binding complexes induced by VIP. In the presence of either $VIP_1R$ antagonist or H89 the supershift patterns returned to the patterns observed for LPS-stimulated cells in the absence of VIP (Fig. 10*B*, *lanes 3* and *4* compared with *lane 1*, and *lanes 8* and *9* compared with *lane 6*). These results suggest

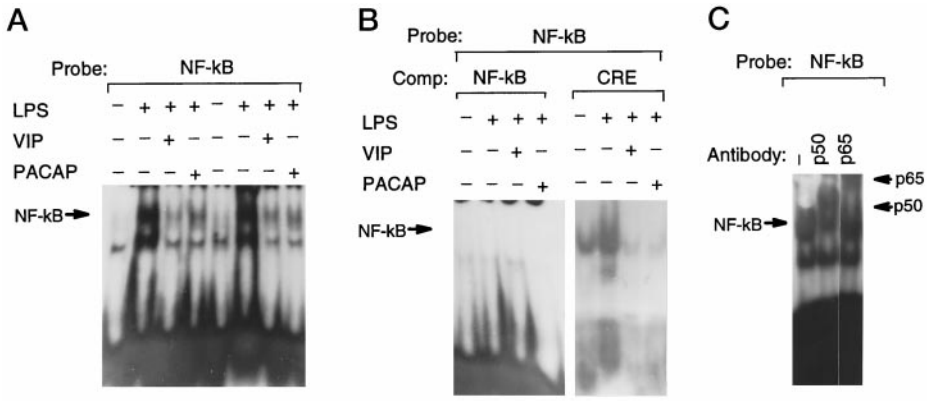


FIG. 7. **VIP and PACAP inhibit NF-kB binding.** *A,* nuclear extracts were prepared from Raw 264.7 cells ($2 \times 10^7$ cells) incubated for 2 h with LPS (1 $\mu$g/ml) in the presence or absence of VIP or PACAP ($10^{-8}$ M). NF-kB binding was assessed by EMSA using a radiolabeled oligonucleotide containing the murine kB site of the TNFα promoter. *B* and *C,* specificity and identification of NF-kB subunit composition was conducted by the addition of 50-fold excess of unlabeled homologous (*NF-kB*) or nonhomologous (*CRE*) oligonucleotides (*Comp*) (*B*), and by supershift analysis using polyclonal antibodies to the p50 and p65 subunits of NF-kB (*C*). The faster migrating band represents nonspecific protein binding. Similar results were observed in three independent experiments.

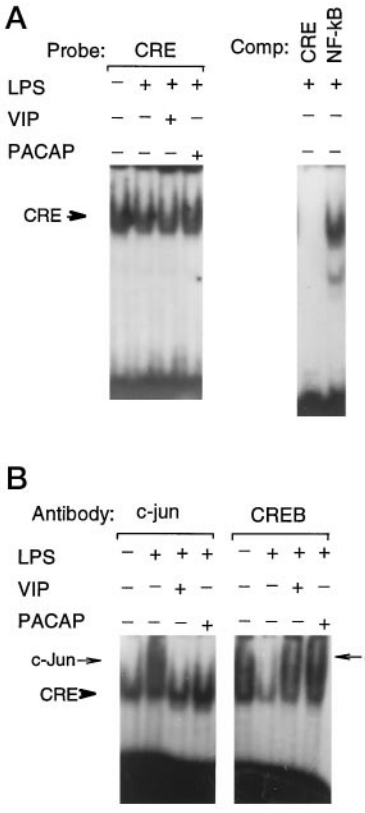


FIG. 8. **VIP and PACAP regulate LPS induction of CRE binding nuclear factors.** *A,* nuclear extracts were prepared from Raw 264.7 cells ($2 \times 10^7$ cells) incubated for 2 h with LPS (1 $\mu$g/ml) in the presence or absence of VIP or PACAP ($10^{-8}$ M). CRE binding activity was determined by EMSA using a radiolabeled oligonucleotide containing the murine CRE site from the TNFα promoter. Specificity was determined by the addition of 50-fold excess of unlabeled nonhomologous (*NF-kB*) or homologous (*CRE*) oligonucleotides (*Comp*). *B,* identification of the proteins bound to the CRE site. Nuclear extracts were preincubated with either anti-c-Jun or anti-CREB antibodies as described under "Experimental Procedures" before the addition of the radiolabeled probe. Similar results were observed in three independent experiments.

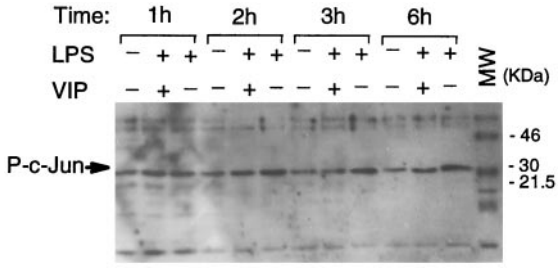


FIG. 9. **VIP and PACAP inhibit LPS-stimulated increases in JNK activity.** Raw 264.7 cells ($2 \times 10^7$ cells) were stimulated with LPS (1 $\mu$g/ml) in the presence or absence of VIP or PACAP ($10^{-8}$ M) for different time periods (1, 2, 3, and 6 h) after which they were lysed and processed as described under "Experimental Procedures." Equal amounts of protein were resolved by SDS-polyacrylamide gel electrophoresis gels and transferred to nitrocellulose membranes. JNK activity was assayed using a specific antibody against phosphorylated c-Jun. Cells cultured in medium served as controls. Phosphorylated c-Jun is indicated by →. *MW,* molecular weight markers. Similar results were observed in three independent experiments.

that both the inhibition of NF-kB and the change in the composition of the CRE binding complexes by VIP are mediated through the $VIP_1R$, but only the change in the CRE binding complex is entirely cAMP-dependent. This is supported by the fact that forskolin (a cAMP inducer) does not affect NF-kB binding (Fig. 10*A*, *lane 5*) but affects CRE binding complexes in the same way as VIP (Fig. 10*B*, *lane 5* compared with *lane 2*, and *lane 10* compared with *lane 7*).

*The cAMP-independent Transduction Pathway Is Involved in the Inhibition of NF-kB Binding*—Because both a cAMP-dependent and a cAMP-independent pathway appear to be involved in the inhibition of TNFα production by VIP, we investigated the possible relationship between the two pathways and the regulation of the kB and CRE binding complexes. Because SNV inhibits TNFα production without the involvement of cAMP, we determined its effect on the kB and CRE binding nuclear factors. Similar to VIP, SNV inhibits NF-kB binding in LPS-stimulated cells, although to a lesser degree (Fig. 11*A*). In contrast, SNV does not change the composition of the CRE binding complexes in LPS-stimulated cells (Fig. 11*B*). This suggests that the cAMP-independent pathway is involved solely in the inhibition of NF-kB binding. This conclusion is supported by the effect of SANV, the lipophilic VIP antagonist that does not affect cAMP induction (22). SANV reverses the inhibitory activity of VIP on NF-kB binding, without affecting the regulatory effect of VIP on the composition of the CRE binding complexes (Fig. 11, *A* and *B*).

## DISCUSSION

VIP and PACAP are two multifunctional neuropeptides with regulatory roles in inflammation (reviewed in Ref. 10). We have recently described that VIP and PACAP protect mice from high

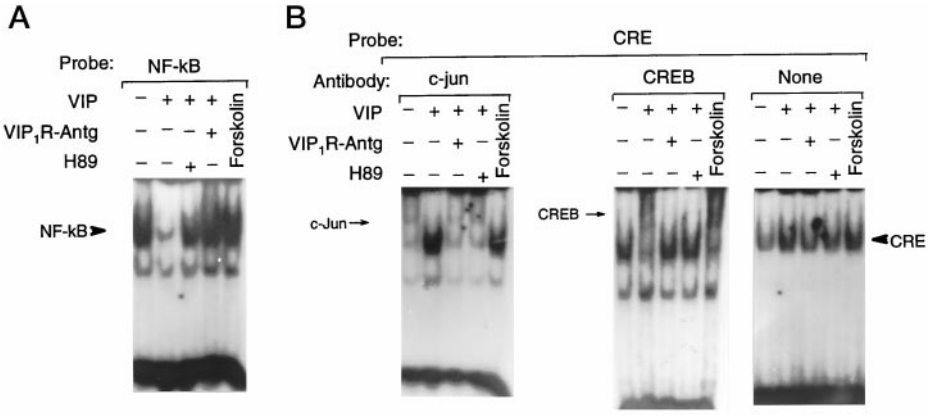


FIG. 10. **Specific receptors and intracellular pathways involved in the VIP and PACAP regulation of nuclear factors.** *A,* NF-kB binding. Nuclear extracts prepared from LPS-stimulated Raw 264.7 cells were incubated with the NF-kB oligonucleotide and subjected to EMSA. *Lane 1*: LPS; *lane 2*: LPS+VIP; *lane 3*: LPS+VIP+H89; *lane 4*: LPS+VIP+$VIP_1R$ antagonist; *lane 5*: LPS+FK. *B,* CRE binding complex. Nuclear extracts prepared from LPS-stimulated Raw 264.7 cells were incubated with the CRE oligonucleotide and subjected to EMSA. In all three panels, *lane 1*: LPS; *lane 2*: LPS+VIP; *lane 3*: LPS+VIP+$VIP_1R$ antagonist; *lane 4*: LPS+VIP+H89; *lane 5*: LPS+FK. *First panel,* supershift with anti-c-Jun Ab; *second panel,* supershift with anti-CREB Ab; *third panel,* no Ab.

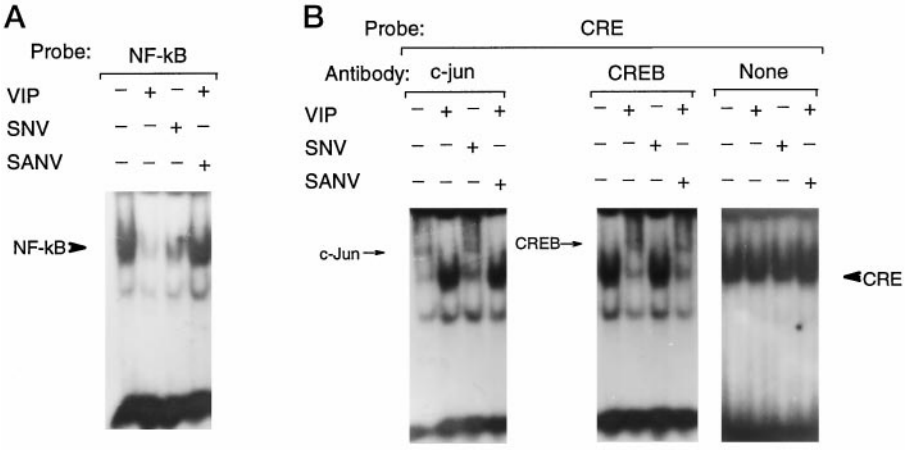


FIG. 11. **Different transduction pathways are involved in the inhibition of NF-kB binding and in the regulation of the composition of CRE binding complexes.** *A,* NF-kB binding. Nuclear extracts prepared from LPS-stimulated Raw 264.7 cells were incubated with the NF-kB oligonucleotide and subjected to EMSA. *Lane 1,* LPS; *lane 2,* LPS+VIP ($10^{-8}$ M); *lane 3,* LPS+SNV ($10^{-7}$ M); *lane 4,* LPS+VIP ($10^{-8}$ M) +SANV ($10^{-6}$ M). *B,* CRE binding complex. Nuclear extracts prepared from LPS-stimulated Raw 264.7 cells were incubated with the CRE oligonucleotide and subjected to EMSA. In all three panels, *lane 1,* LPS; *lane 2,* LPS+VIP ($10^{-8}$ M); *lane 3,* LPS+SNV $10^{-7}$ M); *lane 4,* LPS+VIP ($10^{-8}$ M)+SANV ($10^{-6}$ M). *First panel,* supershift with anti-c-Jun Ab; *second panel,* supershift with anti-CREB Ab; *third panel,* no Ab. One representative experiment of four is shown.

endotoxemia and inhibit *in vitro* and *in vivo* IL-6 and TNFα production by murine peritoneal macrophages (11).[2,3] Here we extend these studies to the molecular mechanisms involved in the inhibitory effect of VIP/PACAP on TNFα production. Our results indicate that VIP/PACAP inhibit LPS-induced TNFα production in Raw 264.7 murine macrophages. The inhibitory effect is dose-dependent within a wide range of neuropeptide concentrations ($10^{-7}$-$10^{-11}$ 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 (10, 41).

Similar to the effect on other cytokines such as IL-2, IL-6, and IL-10 (11, 42, 43), the inhibition of TNFα 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 (44, 45). Peritoneal macrophages were previously shown to express $VIP_1R$ and PACAP-R mRNA, and both high and low affinity VIP/PACAP binding sites (16, 17). Here we report that LPS-stimulated Raw 264.7 macrophages express mRNA for all three VIP/PACAP receptors, although the membrane expression of the three receptors remains to be demonstrated. Our agonist studies suggest that $VIP_1R$ is the major mediator of the VIP/PACAP inhibitory effect on TNFα (60% inhibition observed with the $VIP_1R$ agonist in comparison with 20–25% for $VIP_2R$ and PACAP-R agonists). This is in agreement with Dewit *et al.* (13) who reported a maximal 34% inhibition of TNFα by $10^{-5}$ M Ro 25-1553 (a $VIP_2R$ agonist) in human blood monocytes. If the $VIP_2R$ is expressed only in activated macrophages, as the reverse transcription-PCR results suggest, the lack of effectiveness for the Ro compounds may be because of a lack of appropriate receptors during the early culture period. The role of $VIP_1R$ as the major player in mediating the effect of VIP/PACAP on TNFα production is also supported by the fact that a $VIP_1R$ antagonist, but not PACAP6-38, an antagonist specific for both PACAP-R and $VIP_2R$ (33), reverses the inhibitory effect of VIP/PACAP. Also, the $VIP_1R$ antagonist blocked the effect of VIP/PACAP on both NF-kB and c-Jun/CREB binding to the TNFα promoter, supporting the involvement of the $VIP_1R$ in the regulatory effect of VIP/PACAP on TNFα gene expression.

The $VIP_1R$ is coupled primarily to the adenylate cyclase system (14). TNFα production is inhibited by agents that increase intracellular cAMP levels, and stimulated by the activation of the protein kinase C pathway (2, 4, 46–48). In the present study, H89, a potent and selective inhibitor of protein kinase A reversed the inhibitory effect of VIP/PACAP on TNFα production, suggesting that VIP/PACAP inhibit TNFα production in Raw 264.7 cells through protein kinase A activation and elevation of cAMP levels. However, because the reversal was

[3] M. Delgado, D. Pozo, C. Martinez, J. Leceta, J. R. Calvo, D. Ganea, and R. P. Gomariz, manuscript submitted for publication.

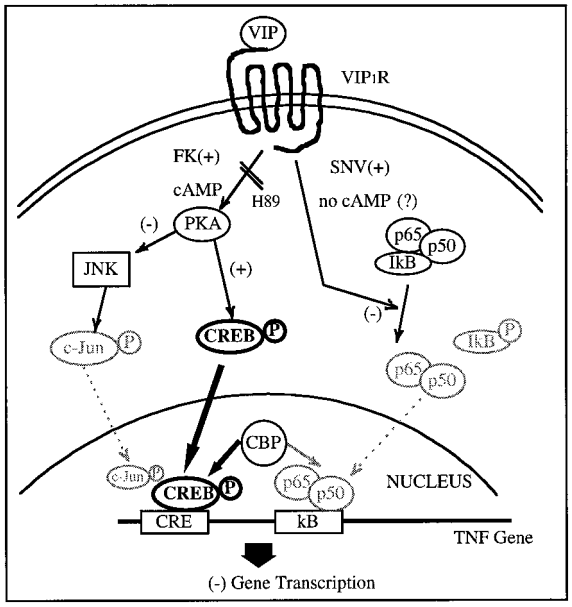


FIG. 12. **Model for the inhibitory effect of VIP/PACAP on TNFα gene expression.** Binding of VIP to $VIP_1R$ initiates two transduction pathways. The cAMP-dependent pathway, mimicked by FK, leads to JNK inhibition and an increase in CREB phosphorylation, resulting in the alteration of the CRE binding complexes from high Jun/low CREB in LPS-stimulated cells to low Jun/high CREB in LPS+VIP-treated cells. The cAMP-independent pathway, mimicked by SNV, leads to an inhibition of NF-kB nuclear translocation, resulting in reduced NF-kB binding. In addition, higher amounts of VIP-induced CREB compete with NF-kB for limited amounts of the coactivator CBP. The decrease in c-Jun and NF-kB, and the sequestering of CBP leads to the inhibition of TNFα transcription.

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 IL-2 and IL-10 production.[4] The existence of a second cAMP-independent pathway is supported by the effect of a lipophilic VIP agonist, SNV, which does not induce cAMP (22). SNV inhibits TNFα production in Raw 264.7 cells, and, as expected in the absence of cAMP induction, the inhibitory effect is not affected by H89. The fact that SNV does not contribute to the inhibitory activity of VIP/PACAP, although it adds to the effect of other cAMP-inducing agents such as $PGE_2$ or forskolin, suggests that indeed a second cAMP-independent pathway may function in the transduction of the VIP/PACAP signal. The nature of this second transduction pathway remains to be determined.

Previous experiments regarding VIP modulation of cytokine expression, indicated different molecular mechanisms, *i.e.* transcriptional regulation for IL-2, IL-6, and IL-10 *versus* post-transcriptional regulation for IL-4 (11, 43, 49). The present study indicates that the inhibitory effect of VIP and PACAP on TNFα production occurs at a transcriptional level. The regulation of the TNFα gene transcription is complex, and involves multiple *cis*-acting elements. Transcriptional regulation by LPS of the TNFα gene has been shown to involve kB sequence motifs and transcriptional factors from the Rel family (18–21, 37, 50, 51). In mammalian cells, the Rel family includes NF-kB1 (p50), RelA (p65), c-Rel, RelB, and NF-kB2 (p50B, p52) (37). NF-kB consists mostly of p50/p65 heterodimers, which are complexed to the inhibitor IkB in the cytoplasm of unstimulated cells; stimuli such as LPS and proinflammatory cytokines induce the phosphorylation of IkB, followed by the release and subsequent nuclear translocation of the p50/p65 heterodimers, which bind to regulatory sequences in a variety of target genes (37). The studies presented here indicate that VIP and PACAP inhibit NF-kB binding in LPS-stimulated Raw 264.7 cells. The NF-kB complex induced by LPS in macrophages was partly supershifted by either anti-p50 or anti-p65 Ab and fully shifted by a combination of these two Abs, suggesting that the NF-kB complex responsible for TNFα induction by LPS consisted of both p50 and p65 subunits. It remains to be determined whether VIP/PACAP-mediated NF-kB nuclear translocation inhibition results from an increase in IkB protein levels, a decrease in IkB degradation, and/or inhibition in IkB phosphorylation, as in the case of other anti-inflammatory agents, such as IL-11, IL-10, transforming growth factor-β1, glucocorticoids, and antioxidants (6, 52–56).

In addition to the kB sites, a CRE site was recently identified as necessary for maximal LPS induction of the TNFα gene in human monocytes (20). Similar to human monocytes, CRE binding activity is constitutively expressed in Raw 246.7 cells. LPS and VIP/PACAP treatment does not increase or decrease the amount of CRE binding; however, supershift experiments with anti-c-Jun Abs indicate that LPS induces a marked increase in c-Jun binding, similar again to human monocytes (20). In contrast, VIP/PACAP reduce c-Jun binding to levels present in the unstimulated cells. This suggests that the inhibitory effect of VIP/PACAP on TNFα gene expression is mediated, at least partially, through a change in the composition of the CRE binding complexes. Because c-Jun phosphorylation by JNK results in both an amplification of c-Jun synthesis and an increase in transactivating activity (40, 57), the effect of VIP/PACAP on c-Jun may be mediated through an effect on JNK. This is indeed the case, because VIP inhibits JNK activity in LPS-stimulated Raw 264.7 cells. The effect of VIP on JNK is in agreement with the previously reported selective inhibition of JNK in T lymphocytes by cAMP-elevating agents (58). In terms of the molecular mechanisms involved in the inhibition of TNFα gene expression, VIP resembles glucocorticoids more than cytokines. IL-10 and IL-11, which inhibit TNFα expression, appear to act solely on NF-kB (6, 52). In contrast, dexamethasone down-regulates both NF-kB and AP-1 binding (59), and this possible synergistic effect may explain why glucocorticoids are more potent TNFα inhibitors.

We investigated the relationship between the cAMP-dependent and cAMP-independent transduction pathways for the inhibitory effect of VIP/PACAP on TNFα expression and the effect on NF-kB and CRE binding activities. Both VIP and forskolin induced similar changes in the CRE binding complexes, and H89, a specific protein kinase A inhibitor, reversed the effect of VIP on the composition of the CRE binding complex. This suggests that VIP/PACAP-elicited cAMP controls changes in the composition of the CRE binding complexes, most probably by increasing protein kinase A-dependent CREB phosphorylation and decreasing JNK-dependent c-Jun phosphorylation. In contrast, increases in cAMP do not appear to directly affect NF-kB binding. Forskolin, a strict cAMP inducer, does not affect NF-kB binding, and H89 reverses only partially the inhibitory effect of VIP. Also SNV, a VIP agonist that does not induce cAMP, inhibits NF-kB binding without affecting the composition of the CRE binding complex. The fact that cAMP-inducing agents do not affect NF-kB binding, although they inhibit kB transcriptional activation, has been previously reported (60). The inhibition of the NF-kB transcriptional activity could result from higher amounts of CRE-bound CREB competing with NF-kB for limiting amounts of the coactivator CBP (61). Recently VIP and PACAP were reported to increase CREB phosphorylation and CREB-regulated tran-

[4] H.-Y. Wang, X. Jiang, I. Gozes, M. Fridkin, D. E. Brenneman, and D. Ganea, manuscript submitted for publication.

scription in several cell types (62–64). Therefore, an additional mechanism in the VIP/PACAP inhibition of kB-mediated transactivation of the TNFα gene may involve the competition between NF-kB and CREB for CBP.

In conclusion, we have shown that the binding of VIP and PACAP to $VIP_1R$ inhibits TNFα production at a transcriptional level in LPS-stimulated Raw 264.7 macrophages through two intracellular pathways, a cAMP-dependent pathway that preferentially increases CREB *versus* c-Jun binding to the CRE site, and a cAMP-independent pathway that inhibits the binding of NF-kB (Fig. 12). The inhibition of TNFα transcription by VIP/PACAP may have significant therapeutic potential, because this proinflammatory cytokine plays a central role in endotoxic shock, multiple sclerosis, cerebral malaria, and various inflammatory diseases (2, 4). Also, the effect of VIP/PACAP on NF-kB binding may be of therapeutic significance, because NF-kB has been proposed as a target for the treatment of inflammatory conditions such as rheumatoid arthritis and inflammatory bowel disease (37).

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