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

(Received for publication, July 9, 1998)

Mario Delgado‡‡, Ernesto J. Munoz-Elias‡, Yanqing Kan‡, Illana Gozes‡, Mati Fridkin‡, Douglas E. Brenneman**, Rosa P. Gomariz‡, and Doina Ganea‡‡ ‡‡

From the ‡Department of Biological Sciences, Rutgers University, Newark, New Jersey 07102, ‡Department of Biologie Cellulaire, Universidad Complutense, Madrid 28040, Spain, §Department of Clinical Biochemistry, Sackler School of Medicine, Tel Aviv University, Tel Aviv 69978, Israel, †Department of Organic Chemistry, Weizmann Institute of Science, Rehovot 76100, Israel, and **Section on Developmental and Molecular Pharmacology, Laboratory of Developmental Neurobiology, NICHD, National Institutes of Health, Bethesda, Maryland 20895

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 264.7 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 VIP1R. VIP/PACAP receptors, agonist and antagonist studies indicate that the major receptor involved is VIP1R. VIP/PACAP inhibit TNFα gene expression by affecting both NF-kB binding and the organization of the cAMP responsive element binding complex (CREB/c-Jun). Two transcription 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 downregulatory 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, 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 endotox shock presumably through the inhibition of TNFα and IL-6 production. 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, VIP1R, and VIP1, 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 VIP1R 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 transcription.

cAMP responsive element; CREB, CRE-binding protein; CBP, CREB-binding protein; PGE2, prostaglandin E2; NF-kB, nuclear factor kB; ELISA, enzyme-linked immunosorbent assay; Ab, antibody; PCR, polymerase chain reaction; bp, base pair(s); H89, N-(2-iodomethyl)-5-iodoquinolinesulfonamide; EMSA, electrophoretic mobility shift assay; FK, forskolin.

‡‡ To whom correspondence should be addressed: Rutgers University, Dept. of Biological Sciences, 101 Warren St., Newark, NJ 07102. Tel.: 973-353-1162; Fax: 973-353-1007; E-mail: dganea@andromeda.rutgers.edu.

* 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; PGE2, prostaglandin E2; NF-kB, nuclear factor kB; ELISA, enzyme-linked immunosorbent assay; Ab, antibody; PCR, polymerase chain reaction; bp, base pair(s); H89, N-(2-iodomethyl)-5-iodoquinolinesulfonamide; EMSA, electrophoretic mobility shift assay; FK, forskolin.

** Delgado, M., Martinez, C., Pozo, D., Receta, 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 TNF-α 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, VIP1-32, and VIP10-28 were purchased from Novabiochem (Lauf, Germany, Switzerland). The VIP, PACAP-27, and PACAP-38 (henceforth referred to as VIP, the VIP-R-agonist) (Lys1-4Arg5Leu6VIP(1-4)GRF(8-27) [16]), the VIP-R-agonist (Lys14Arg15Leu16VIP(1-4)GRF(8-27)) were kindly donated by Dr. Patrick Robberecht (Université Libre de Bruxelles, Belgium). The VIP-R-agonists Ro 25-1392 Ac-Gluu-OCH3-TyrLys4-Asp10Leu20-Lys25-VIP-25 (21:25) and Ro 25-1533 Ac-Gluu-Lys4-Asp10Leu20-Lys25-VIP25 (21-25) were generous gifts from Drs. Ann Welton and David R. Bolin (Hoffmann-La Roche). The PACAP-R agonist maxadilan (does not cross-react with PACAP-R) was donated by Dr. Patrick Robberecht (Universite Libre de Bruxelles, Belgium). The prehybridization and hybridization buffers were purchased from Boehringer Mannheim (Mannheim, Germany). All the oligonucleotides were synthesized, annealed, and end-labeled with [32P]dATP. The probes for the nuclear DNA were labeled by nick-translation with [32P]dATP. The probes for the nuclear DNA were labeled by nick-translation with [32P]dATP. The probes were incubated on ice for 30 min on ice in 0.1 M sodium phosphate, pH 7.2, 0.4 M NaCl, 1 mM EDTA, 0.1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 5 mM NaF, 1 mM Na3VO4, and 1 mM Na4P2O7. Supernatants containing nuclear proteins were harvested 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 M buffer C (20 mM HEPES, pH 7.5, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 0.1 M dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 1 mM Na4P2O7). Supernatants containing nuclear proteins were harvested by centrifugation at 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 (EMSA).

**Inhibition of LPS-induced TNF-α production by VIP/PACAP**—To investigate whether VIP/PACAP inhibit TNF-α 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 TNF-α released in the culture supernatants was assayed by ELISA at different time periods. VIP and PACAP inhibit the TNF-α 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).

**RESULTS**

VIP and PACAP Inhibit LPS-induced TNF-α Production by Raw 264.7 Macrophages—To investigate whether VIP/PACAP inhibit TNF-α 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 TNF-α released in the culture supernatants was assayed by ELISA at different time periods. VIP and PACAP inhibit the TNF-α 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).
Inhibition of TNFα by VIP/PACAP Molecular Mechanisms

was observed with various concentrations of glucagon (10 to 1000 ng/ml) and PACAP molecules are required for their inhibitory activity (32) on the LPS-induced TNFα production. VIP, VIP-R, VIP₂R, and PACAP-R agonists inhibited TNFα release (Fig. 2B). The VIP-R 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. 2B). In addition, we investigated the ability of PACAP 8–38, an antagonist specific for PACAP-R and VIP₂R (33), and of a specific VIP₂R-antagonist (34), to reverse the effects of VIP and PACAP. Increasing concentrations of the antagonists (10⁻⁸–10⁻⁶ M) were added simultaneously with a fixed concentration of VIP or PACAP (10⁻⁶ M). The inhibitory effects of VIP and PACAP were reversed by the VIP₂R-antagonist in a dose-dependent manner (Fig. 2C). In contrast, PACAP 8–38 did not reverse the inhibitory effect of VIP or PACAP (Fig. 2D). Neither the VIP₂R-antagonist nor PACAP 8–38 significantly affected TNFα levels (Fig. 2, C and D legends). Furthermore, the simultaneous addition of VIP or PACAP and VIP₁R-agonist did not result in an additive effect on TNFα release (Fig. 2E). Together these results confirm the specificity of the VIP and PACAP inhibitory activity and suggest that both neuropeptides exert their action through binding to VIP₁R.

Finally, the simultaneous addition of VIP and 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 VIP₂R agonists before the addition of VIP (Fig. 2F).

Raw 264.7 Cells Express VIP₁R, VIP₂R, and PACAP-R—The fact that VIP₁R, VIP₂R, 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 VIP₁R, VIP₂R, and PACAP-R mRNA by reverse transcription-PCR in unstimulated and LPS-stimulated Raw 264.7 cells. Both VIP₁R- and PACAP-R-specific fragments were amplified from unstimulated and stimulated macrophages, whereas VIP₂R fragments were only detected in stimulated cells (Fig. 3). These results indicate that LPS-stimulated Raw 264.7 cells express VIP₁R, VIP₂R, and PACAP-R mRNA.

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 10⁻⁶ 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.

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 VIP₁R-agonist (29), of two VIP₂R 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. VIP₁R, VIP₂R, and PACAP-R agonists inhibited TNFα release (Fig. 2B). The VIP₁R 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. 2B). In addition, we investigated the ability of PACAP 8–38, an antagonist specific for PACAP-R and VIP₂R (33), and of a specific VIP₂R-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 (10⁻⁶ M). The inhibitory effects of VIP and PACAP were reversed by the VIP₂R-antagonist in a dose-dependent manner (Fig. 2C). In contrast, PACAP 8–38 did not reverse the inhibitory effect of VIP or PACAP (Fig. 2D). Neither the VIP₂R-antagonist nor PACAP 8–38 significantly affected TNFα levels (Fig. 2, C and D legends). Furthermore, the simultaneous addition of VIP or PACAP and VIP₁R-agonist did not result in an additive effect on TNFα release (Fig. 2E). Together these results confirm the specificity of the VIP and PACAP inhibitory activity and suggest that both neuropeptides exert their action through binding to VIP₁R.

Finally, the simultaneous addition of VIP and 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 VIP₂R agonists before the addition of VIP (Fig. 2F).

Raw 264.7 Cells Express VIP₁R, VIP₂R, and PACAP-R—The fact that VIP₁R, VIP₂R, 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 VIP₁R, VIP₂R, and PACAP-R mRNA by reverse transcription-PCR in unstimulated and LPS-stimulated Raw 264.7 cells. Both VIP₁R- and PACAP-R-specific fragments were amplified from unstimulated and stimulated macrophages, whereas VIP₂R fragments were only detected in stimulated cells (Fig. 3). These results indicate that LPS-stimulated Raw 264.7 cells express VIP₁R, VIP₂R, and PACAP-R mRNA.

Intracellular Signal Pathways Involved in the Inhibitory Activity of VIP and PACAP on TNFα Production—aCAMP 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. 4C); 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. 5A), and the inhibitory activity is not...
FIG. 2. Inhibition of TNFα production by VIP and PACAP is specific and is mediated through VIP1R. 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, VIP1–12, and VIP10–28 (A), or maxadilan (PACAP-R-agonist), Ro 25-1632, Ro 25-1553 (VIP1R-agonists), or (Lys15,Arg16,Leu27)VIP(1–7)-GRF(8–27) (VIP1R-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 VIP1R 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 VIP1R-antagonist (Ac-His1,D-Phe2,Lys15,Arg16,Leu27)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. VIP1R antagonist and PACAP6–38 did not affect TNFα levels (1577 ± 112 pg/ml for 10–8 M VIP1R antagonist; 1516 ± 145 pg/ml for 10–7 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 < 0.01, and *** p < 0.001 compared with samples treated with neuropeptides and without antagonists.

E and F, effect of VIP1R, VIP2R, and PACAP-R agonists. Raw 264.7 cells were stimulated with LPS (1 μg/ml) and treated with a VIP1R 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. 5B). 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 E2 (PGE2), two strict cAMP-inducing agents. Although SNV did not significantly affect the VIP/PACAP inhibitory effect, it increased the inhibitory action of forskolin and PGE2 (Fig. 5C). 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, PGE2, and 8-Br-cAMP. SANV partially reverses the inhibitory effect of VIP, PACAP, forskolin, PGE2, and 8-Br-cAMP (Fig. 5D), 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. 7A). The binding specificity was confirmed by using homologous (NF-kB) and nonhomologous (CRE) oligonucleotides as competitors (Fig. 7B). 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. 7C).

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. 8A). The specificity of the CRE binding activity was confirmed with homologous (CRE) or nonhomologous (NF-kB) oligonucleotides as competitors (Fig. 8A). 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. 8B). In contrast, a major supershift by the anti-c-Jun Ab was evident in cells treated with LPS (Fig. 8B), 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. 8B).

VIP and PACAP Inhibit JNK Activity in LPS-stimulated Raw 264.7 Cells—Phosphorylation of c-Jun at Ser⁶³ and Ser⁷³ 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,R, VIP,R, and PACAP-R mRNA in Raw 264.7 cells. Total RNA extracted from unstimulated macrophages (lane 2) and LPS-stimulated macrophages (lane 3) (2 × 10⁶ cells) was subjected to reverse transcription-PCR with specific primers for VIP,R, VIP,R, 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 of 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.
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, R 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 R and cAMP, we determined the effect of the VIP R 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 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 R 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

---

**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) and incubated with VIP (10−8 M), PACAP (10−8 M), FK (10−6 M), or PGE2 (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 TNF/μl). Numbers in parenthesis represent expected percentages of inhibition if the effects of SNV and other agents were additive. Each result is the mean ± S.D. of four experiments performed in duplicate. D, 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), 8-Br-cAMP (10−5 M), or PGE2 (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 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.

---

**VIP and PACAP inhibit TNFα transcription.** Raw 264.7 cells (2 x 10^7 cells) were stimulated with LPS (1 μg/ml) and incubated with or without VIP or PACAP (10−6 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.
that both the inhibition of NF-kB and the change in the composition of the CRE binding complexes by VIP are mediated through the VIP1R, 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. 10A, lane 5) but affects CRE binding complexes in the same way as VIP (Fig. 10B, 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 results were observed in three independent experiments.

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
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 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 (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 VIP1R 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 VIP1R is the major mediator of the VIP/PACAP inhibitory effect on TNFα (60% inhibition observed with the VIP1R agonist in comparison with 20–25% for VIP2R 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 VIP1R agonist) in human blood monocytes. If the VIP1R 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 VIP1R as the major player in mediating the effect of VIP/PACAP on TNFα production is also supported by the fact that a VIP1R antagonist, but not PACAP6-38, an antagonist specific for both PACAP-R and VIP2R (33), reverses the inhibitory effect of VIP/PACAP. Also, the VIP1R 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 VIP1R in the regulatory effect of VIP/PACAP on TNFα gene expression.

The VIP1R 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 C 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...
Inhibition of TNFα by VIP/PACAP Molecular Mechanisms

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 transcription.

---

**Fig. 12. Model for the inhibitory effect of VIP/PACAP on TNFα gene expression.** Binding of VIP to VIPR 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. 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 PGE2 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 posttranscriptional 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 IκB in the cytoplasm of unstimulated cells; stimuli such as LPS and proinflammatory cytokines induce the phosphorylation of IκB, 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 IκB protein levels, a decrease in IκB degradation, and/or inhibition in IκB 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.
Inhibition of TNFα by VIP/PACAP Molecular Mechanisms

In conclusion, we have shown that the binding of VIP and PACAP to VIP-R 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-κB (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-κB binding may be of therapeutic significance, because NF-κB has been proposed as a target for the treatment of inflammatory conditions such as rheumatoid arthritis and inflammatory bowel disease (37).

Acknowledgments—We thank Dr. David Pozo (University of Sevilla, Sevilla, Spain) for the TNFα oligonucleotide probe, Dr. Patrick Robberecht (Université Libre de Bruxelles, Brussels, Belgium) for the VIP-R agonist and antagonist, Drs. David Bolin and Ann Welton (Hoffman-LaRoche, Inc.) for the VIP-R agonist Ro 25-1553, and Dr. Ethan Lerner (Massachusetts General Hospital, Charlestown, MA) for the PACAP-R agonist maxadilan.

REFERENCES

1. Laskin, D. L., and Pendino, K. J. (1995) Annu. Rev. Pharmacol. Toxicol. 35, 655–677
2. Beutler, B., and Cerami, A. (1989) Annu. Rev. Immunol. 7, 625–655
3. Van Snick, J. (1990) Annu. Rev. Immunol. 8, 253–278
4. Vassalli, P. (1992) Annu. Rev. Immunol., 10, 411–452
5. Kunkel, S. L., Spengler, M., May, M. A., Spengler, R., Larrick, J., and Remick, D. (1988) J. Biol. Chem. 263, 5380–5384
6. Treplicchio, W., Bozza, M., Pedeneaux, G., and Dorner, A. (1996) J. Immunol., 157, 3627–3634
7. Fiorentino, D. F., Zlotnik, A., Mosmann, T. R., Howard, M., and O’Garra, A. (1991) J. Immunol. 147, 3815–3822
8. Tsuwanuki, S., Sporn, M., Ding, A., and Nathan, C. F. (1988) Nature 334, 260–262
9. Muchamuel, T., Menon, S., Pisacane, P., Howard, M. C., and Cockayne, D. A. (1991) J. Immunol. 146, 2898–2903
10. De la Fuente, M., Delgado, M., and Gomariz, R. P. (1996) Adv. Neuroimmunol. 6, 75–91
11. Martinez, C., Delgado, M., Pozo, D., Lecca, J., Calvo, J. R., Ganea, D., and Gomariz, R. P. (1998) J. Leukocyte Biol. 63, 591–601
12. Hernandez, A., Tato, E., De la Fuente, M., de Miguel, E., and Arnalich, F. (1996) J. Neuroimmunol. 71, 25–30
13. Dewil, D., Gourlet, P., Formisano, S., Venuta, S., and Turco, M. C. (1996) J. Immunol. 58, 57–60
14. Rawling, S. R., and Hezareh, M. (1996) Endo. Rev. 17, 4–29
15. Segura, J. J., Guerrero, J. M., Gomariz, R. P., and Calvo, J. R. (1991) Regul. Pept. 33, 133–143
16. Delgado, M., Pozo, D., Martinez, C., Garrido, E., Lecca, J., Calvo, J. R., and Gomariz, R. P. (1990) Regul. Pept. 32, 161–166
17. Pozo, D., Delgado, M., Martinez, C., Garrido, E., Lecca, J., Calvo, J. R., and Gomariz, R. P. (1990) J. Biol. Chem. 265, 10005–10016
18. Shakhov, A. N., Collart, M. A., Vassalli, P., and Viallard, J.-P. (1996) Mol. Cell. Biol. 16, 1057–1065
19. Shakhov, A. N., Collart, M. A., Vassalli, P., Nedospasov, S. A., and Jorgensen, C. V. (1993) J. Exp. Med. 171, 35–47
20. Yao, J., Mackman, N., Edgington, T. S., and Fan, S. (1997) J. Biol. Chem. 272, 17795–17801
21. Trede, N. S., Tsutsuykova, A. V., Chatila, T., Goldfeld, A. E., and Geha, R. S. (1995) J. Immunol. 155, 902–908
22. Gozes, I., Lilling, G., Glazer, R., Ticher, A., Ashkenazi, I. E., Davidson, A., Rubinrart, S., Fridkin, M., and Brenneman, D. E. (1995) J. Pharmacol. Exp. Ther. 273, 161–167
23. Fransen, L., Mueller, R., Marmont, A., Tavener, J., Van der Heyden, J., Kawashima, E., Collet, A., Tizard, R., Van Heusden, W., Van Vliet, A., Boesveldt, H. R., and Fiers, W. (1985) Nucleic Acids Res. 13, 4417–4422
24. Inagaki, N., Yoshida, H., Mizuta, M., Gono, T., Miyazaki, J. I., and Seino, S. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 2679–2683
25. Hashimoto, H., Yamamoto, K., Hagigana, N., Ogawa, N., Nishimo, A., Aino, H., Nogi, H., Inamishi, K., Matsuda, T., and Baba, A. (1996) Biochem. Biophys. Acta 1281, 129–133
26. Johnson, M., McCormack, R., Delgado, M., Martinez, C., and Ganea, D. (1996) J. Neuroimmunol. 68, 27–38
27. Schreiber, K., Mettias, P., Muller, M. M., and Shaffer, W. (1989) Nucleic Acids Res. 17, 6419
28. Gourlet, P., Vandermeers-Piret, M. C., Calvo, J. R., and Hezareh, M. (1996) J. Immunol. 156, 109–119
29. Gourlet, P., Vandermeers-Piret, M. C., Calvo, J. R., and Hezareh, M. (1996) J. Pharmacol. Exp. Ther. 282, 1–11
30. Marcou, J., and Lerner, E. A. (1997) J. Biol. Chem. 272, 966–970
31. Martinez, C., Delgado, M., Pozo, D., Gomariz, R. P., and Ganea, D. (1996) Eur. J. Pharmacol. 287, 7–11
32. Gourlet, P., De Neef, P., Calvo, J. R., Caubel, M., and Robberecht, P. (1997) Peptides 18, 1535–1545
33. Martine, C., Pozo, D., Martinez, C., Calvo, J. R., and Ganea, D. (1996) Immunol. Lett. 50, 1498–1506
34. Xia, M., Sreedharan, S. P., Bolin, D. R., Gaio, G. O., and Goetzl, E. J. (1997) J. Pharmacol. Exp. Ther. 281, 629–633
35. Ouro, M., and Lerner, E. A. (1997) J. Biol. Chem. 272, 966–970
36. Gourlet, P., Vandermeers-Piret, M. C., Rathe, J., De Neef, P., and Robberecht, P. (1997) Surgery 122, 204–211
37. Gourlet, P., De Neef, P., Calvo, J. R., Caubel, M., and Robberecht, P. (1997) Peptides 18, 1556–1560
38. Kobayashi, E., Nakho, H., Morimoto, M., and Tamaoki, T. (1989) Biochem. Pharmacol. 3815–3822