Vasoactive Intestinal Peptide and Pituitary Adenylate Cyclase-activating Polypeptide Inhibit Nuclear Factor-κB-dependent Gene Activation at Multiple Levels in the Human Monocytic Cell Line THP-1*

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The neuropeptides vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating polypeptide (PACAP) suppress monocyte/macrophage production of proinflammatory agents. The transcription factor NF-κB regulates the transcription of most agents. VIP/PACAP inhibit NF-κB transactivation in the lipopolysaccharide-stimulated human monocytic cell line THP-1 at multiple levels. First, VIP/PACAP inhibit p65 nuclear translocation and NF-κB DNA binding by stabilizing the inhibitor IκBα. Second, VIP/PACAP induce phosphorylation of the CRE-binding protein (CREB) and its binding to the CREB-binding protein (CBP). This results in a decrease in p65-CBP complexes, which further reduces NF-κB transactivation. Third, VIP and PACAP reduce the phosphorylation of the TATA box-binding protein (TBP), resulting in a reduction in TBP binding to both p65 and the TATA box. All these effects are mediated through the specific receptor VPAC1. The cAMP/cAMP-dependent protein kinase pathway mediates the effects on CBP and TBP, whereas a CAMP-independent pathway is the major transducer for the effects on p65 nuclear translocation. Since NF-κB represents a focal point for various stimuli and induces the expression of many proinflammatory genes, its targeting by VIP and PACAP positions them as important anti-inflammatory agents. The VIP/PACAP inhibition of NF-κB at various levels and through different transduction pathways could offer a significant advantage over other anti-inflammatory agents.

Human monocytes can be induced to express a variety of genes involved in immune and inflammatory responses and cell adhesion. Following stimulation with microbial products like lipopolysaccharide (LPS),1 monocytes secrete and/or express several inflammatory factors such as TNFα, IL-12, IL-1, IL-6, nitric oxide, granulocyte-macrophage colony-stimulating factor, chemokines such as IL-8, RANTES (regulated on activation normal T cell expressed), macrophage chemotactic protein-1 and macrophage inflammatory protein-1α/β, the adhesion molecule ICAM-1, and the stimulatory complex for T cells, i.e. B7 and MHC class II molecules.

The pleiotropic transcription factor NF-κB plays an important role in the transcriptional regulation of all these genes (reviewed in Ref. 1). NF-κB occurs in both homo- and heterodimeric forms. The most common transcriptionally active form is a p50/p65 heterodimer (reviewed in Ref. 2). In unstimulated cells, NF-κB is localized in the cytosol bound to inhibitor proteins, collectively termed IκB. Stimulation results in IκB phosphorylation, ubiquitination, and proteosomal degradation, followed by the rapid translocation of NF-κB to the nucleus where it binds to specific κB elements within promoters (1, 2).

Several studies have shown that the transactivating activity of NF-κB requires DNA binding and interaction with coactivators that bridge various transcriptional activators and components of the basal transcriptional machinery. The CREB-binding protein (CBP) is a ubiquitously expressed nuclear coactivator present in limiting amounts (reviewed in Ref. 3). A diverse and increasing number of transcription factors and some elements of the basal transcriptional machinery are able to form stable physical complexes with and respond to CBP (reviewed in Refs. 4 and 5). CBP functions as an integrator linking various transcription factors to the basal transcriptional apparatus, by binding to the basal transcription factor TFIIB, which in turn contacts the TATA box-binding protein (TBP) of the TFIID complex in the basal apparatus (6, 7). The interaction of p65 with CBP is essential for NF-κB transcriptional activity (8, 9), and this interaction can be strengthened by p65 phosphorylation (6, 10), or impeded by competition from other CBP-binding factors such as CREB, c-Jun, c-Fos, p53, steroid receptors, c-Myb, and Myo-D (7, 11–13).

Vasoactive intestinal peptide (VIP) and the structurally related pituitary adenylate cyclase-activating polypeptide (PACAP), two neuropeptides present in the lymphoid microenvironment, elicit a broad spectrum of biological functions, including the modulation of innate and adaptive immunity (receptor; TNFα, tumor necrosis factor; ELISA, enzyme-linked immunosorbent assay; Ab, antibody; HA, hemagglutinin; PAGE, polyacrylamide gel electrophoresis; RT-PCR, reverse transcriptase-polymerase chain reaction; EMSA, electrophoretic mobility shift assay; PKA, cAMP-dependent protein kinase; H89, N-[2-(p-bromocinnamyl-amo)ethy]-5-iso-quinolinesulfonamide; EGFP, enhanced green fluorescent protein; HMG, high mobility group.

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The abbreviations used are: LPS, lipopolysaccharide; CBP, CREB-binding protein; CREB, cAMP regulatory element-binding protein; IKK, IκB kinase; IL, interleukin; MAPK, mitogen-activated protein kinase; MEKK1, MEK kinase 1; MEK, MAPK kinase; NF-κB, nuclear factor κB; PACAP, pituitary adenylate cyclase activating polypeptide; PAC1, PACAP receptor; TBP, TATA box binding protein; VIP, vasoactive intestinal peptide; VPAC1, type 1 VIP receptor; VPAC2, type 2 VIP.
viewed in Refs. 14–17). VIP and PACAP down-regulate the innate response by inhibiting inducible nitric-oxide synthase expression and secretion of pro-inflammatory cytokines in stimulated macrophages (18–23). VIP and PACAP affect the adaptive T cell response indirectly, by down-regulating B7.1/B7.2 expression and the subsequent costimulatory function of activated macrophages (24) and directly by inhibiting IL-2 production and T cell proliferation (reviewed in Ref. 15). Many of the proinflammatory cytokines and costimulatory proteins affected by VIP and PACAP are known to be regulated by NF-κB (1). In fact, we have previously demonstrated that VIP and PACAP inhibit NF-κB nuclear translocation and DNA binding to several promoters in both murine macrophages and T cells (18, 25–27). However, the effect of VIP and PACAP on NF-κB activation in human monocytes has not been investigated to date. In addition, although we showed that VIP and PACAP inhibit NF-κB DNA binding, a direct effect on NF-κB-dependent transcriptional activity has not yet been addressed. Furthermore, we also asked whether VIP and PACAP could regulate NF-κB transcriptional activity through the regulation of coactivators. Our data show that VIP and PACAP decrease NF-κB-dependent transcriptional activity in the LPS-stimulated human monocytic cell line THP-1. This effect is exerted at multiple levels. The neuropeptides inhibit NF-κB nuclear translocation and DNA binding by inhibiting the IκB kinase (IKK)-mediated IκB phosphorylation/degradation. In addition, VIP and PACAP selectively inhibit the interaction of p65 with CBP, while increasing interactions between CBP and CREB. Furthermore, by inhibiting the LPS-induced MEKK1/MEK3/MEK6/p38 MAPK pathway, the two neuropeptides inhibit TBP activation and its subsequent DNA binding and interaction with p65. The differential involvement of specific VIP/PACAP receptors and intracellular pathways was also addressed. The specific receptor VPAC1 mediates the effects of VIP/PACAP on p65 nuclear translocation, formation of p65-CBP complexes and TBP activation and formation of p65-TBP complexes. However, whereas a CAMP-independent pathway is primarily responsible for the effects on p65 translocation, the cAMP/PKA pathway mediates the effects on the availability and/or activation of the coactivators CBP and TBP.

EXPERIMENTAL PROCEDURES

Reagents—Synthetic VIP, PACAP-(38), and SB 203580 were purchased from Calbiochem. The PAC1/VPAC2-antagonist PACAP-(22–38) was obtained from Peninsula Laboratories (Belmont, CA). The VPAC1-antagonist [Ac-His<sub>5</sub>,Phe<sub>1</sub>Lys<sub>5</sub>Arg<sub>5</sub>Leu<sub>9</sub>](VIP-(3–7)-GRF-(8–27)] was kindly donated by Dr. Patrick Robberecht (Universite Libre de Bruxelles, Belgium). Human recombinant TNFα and capture and biotinylated antibodies against human TNFα were purchased from Pharmingen (San Diego, CA). LPS (from Escherichia coli 055:B5), DEAE-dextran, protease inhibitors, and forskolin were purchased from Sigma, and N-(p-bromocinnamylamino)ethyl-5-isouquinolin sulfonamide (H89) was from ICN Pharmaceuticals Inc. (Costa Mesa, CA). Recombinant IκBα (1–317) and TFIID (TBP)-tagged fusion proteins and antibodies against p65, p50, IκBα, IκB-kinase α (IKKα), CREB, p38 MAPK, MEKK1, MEK3, MBP, NF-κB (CBF-A), HMG-Y(I), phosphorylated IκBα, and NF-ATp were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against phosphorylated-p38 MAPK, phosphorylated MEKK3/6, and phosphorylated-CREB were purchased from New England Biolabs (Beverly, MA).

Cells—THP-1, a human leukemic monocytic cell line, was obtained from American Type Culture Collection (Manassas, VA). THP-1 cells were stimulated with LPS (0.5 μg/ml) or TNFα (20 ng/ml) in the presence or absence of VIP or PACAP (38) (10<sup>–8</sup> M unless mentioned otherwise).

Plasmids, Transfections, and Luciferase Assay—NF-κB-dependent gene expression was evaluated using a luciferase reporter gene driven by four tandem copies of the κ enhancer (κE<sub>b</sub>) in a pUC vector (CLONTECH, Palo Alto, CA). CRE-dependent gene expression was evaluated using a luciferase reporter gene (CLONTECH). The plasmid pRc/RSVp65 containing the entire cDNA of p65 was kindly provided Drs. G. J. Nabel and J. Stein through the National Institutes of Health AIDS Research and Reference Reagent Program. The expression plasmid, pRc/RSV-mCBP.HA.RK containing the full-length mouse CBP cDNA with a hemagglutinin (HA) tag (6) was a generous gift of Dr. R. Goodman. IκBα degradation was assayed by transiently transfecting an enhanced green fluorescent protein (EGFP)-tagged IκBα (IκBα-EGFP) plasmid (CLONTECH) followed by flow cytometry analysis following the manufacturer's recommendations. Empty vectors pRc/RSV and pUC-18 (Invitrogen, Carlsbad, CA) were used to maintain a constant total DNA concentration in each experiment. To assess variations in transfection efficiencies, the cells were transfected with 2 μg of the control plasmid pSV-β-galactosidase (Baltimore Biologicals) that expresses β-galactosidase. Levels of β-galactosidase were determined using the Galacto-Light assay system (Tropix Inc., Bedford, MA) and exhibited <15% variation between samples.

THP-1 cells were transiently transfected with a total of 10–30 μg of plasmid DNA using DEAE-dextran. Forty eight hours later, the cells were stimulated with LPS (500 ng/ml) in the absence or presence of VIP or PACAP, and 6 h later luciferase assays were carried out as recommended by the manufacturer (Promega). Luciferase activity, expressed in arbitrary light units, was corrected for protein concentration or normalized to coexpressed β-galactosidase levels.

RNA Extraction and Northern Blot Analysis—Northern blot analysis was performed according to standard methods. The probe for human TNFα was generated by RT-PCR as described previously using the following primers: TNFα-5′-GGCTTCAGGCTCTCTTCCT-3′ and 5′-ATCTATCTGGGAGGGGTCTT-3′ (28). Signal quantitation was performed in a PhosphoImager SI (Molecular Dynamics, Sunnyvale, CA).

Electrophoretic Mobility Shift Assay (EMSA)—Nuclear extracts were prepared by the mini-extraction procedure of Schreiber et al. (29). Double-stranded oligonucleotides (50 ng) corresponding to the human nuclear factor NF-κB site (5′-AGTGGAGGGGGTTCCTCCAGG-3′) (Promega), TFIIID (TBP) (5′-CGAGGACCATATAAA TGAGGTAGGAA-3′, Santa Cruz Biotechnology), and the consensus CRE motif (5′-AGAGATTGCCTGACGCTAGAGCTAG-3′, Santa Cruz Biotechnology) were end-labeled with [γ-<sup>32</sup>P]ATP. For EMSAs with THP-1 nuclear extracts, 20,000–50,000 cpm of double-stranded oligonucleotides, corresponding to ~0.5 μg, were used. The reaction mixtures (15 μl) were set up containing 0.1–0.5 ng of DNA probe, 5 μg of polyclonal- or monoclonal-antibodies to NF-κB, 2 μg of poly(d-I-dC)-poly(d-I-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). The mixtures were incubated on ice for 15 min before adding the probe, followed by another 20 min at room temperature. Samples were subjected to electrophoresis in 4% non-denaturing polyacrylamide gels. In competitive antibody experiments, the nuclear extracts were incubated for 15 min at room temperature with specific antibodies (1 μg) or competing cold oligonucleotides (50-fold excess) before the addition of the labeled probe.

Immunoprecipitation Experiments and Western Blotting—For Western blot analysis, whole cell lysates, cytoplasmic fraction, or nuclear extract (see above) containing 20–30 μg of protein were subjected to SDS-PAGE (12.5%). After electrophoresis and electroblotting the membranes were developed with the enhanced chemiluminescence detection system (ECL, Amersham Pharmacia Biotech).

Phosphorylated MEK3 and MEK6 were detected by immunoprecipitation with anti-MEK3 or anti-MEK6 Abs, followed by SDS-PAGE and immunoblotting with an Ab against phosphorylated MEK3/6.

Interaction of CBP or TBP with p65 and/or CREB was assessed by immunoprecipitation of cell extracts (200 μg) with 1–2 μg of anti-p65 or anti-CREB antibodies, followed by treatment with 25 μl of protein A/G-Sepharose beads (Sigma). After extensive washing and boiling in 1× SDS sample buffer, the complexes were subjected to Western blotting with anti-CBP or anti-TBP antibodies. For transient transfections with the CBP vector, we used an anti-hemagglutinin Ab (anti- HA; Roche Molecular Biochemicals) to detect transfected HA-tagged CBP. After detection with an appropriate secondary antibody-conjugated peroxidase, proteins were visualized by enhanced chemiluminescence.

In Vitro Kinase Assays—In vitro kinase assays were performed as described previously with some modifications (30). Endogenous IKKα and p38 MAPK were immunoprecipitated from lysates (200 μg) by incubation of 0.5 μl of antibody (anti-IKKα or anti-p38 MAPK) antibodies, for 2 h at 4 °C. The immune complexes were harvested with protein A/G-Sepharose for 45 min at 4 °C. The beads were extensively washed and resuspended in 30 μl of kinase buffer with 15 μg ATP, 10 μCi of [γ-<sup>32</sup>P]ATP (3000 Ci/mmol), containing 5 μg of recombinant IκBα (for IKKα) or TBP (for p38 MAPK). The kinase reaction was performed at 30 °C for 30 min and stopped by the addition of 15 μl of 2× SDS.
sample buffer. Following boiling for 5 min, the samples were subjected to SDS-PAGE (9%), electroblotting, and autoradiography.

**Immunoblotting of Proteins Bound to the Proximal Region of the TNFα Promoter**—A double-stranded oligonucleotide spanning the proximal region of the human TNFα promoter (−661 to −1), generated from THP-1 genomic DNA by PCR (primers used, 5′-TCAGAAGTTAAA GAGGCCGTC-3′ and 5′-GGCTGGGTGTGCCAAACACT-3′) and biotinylated in our molecular biology facility, was coupled to Dynabeads M-280 streptavidin (Dynal, Lake Success, NY) according to the manufacturer's recommendations. The TNFα promoter-coupled matrix (250 μg) was incubated with 25 μl of THP-1 nuclear extract in binding buffer (50 mM NaCl, 5 mM MgCl2, 10 mM Tris, pH 7.5, 1 mM dithiothreitol, 1 mM EDTA, 0.25 μg/ml poly(dI)poly(dC), and 5% glycerol) for 20 min at room temperature while mixing every 5 min to keep the Dynabeads in suspension. The flow-through fractions were collected; the Dynabeads were washed four times with binding buffer containing 0.5 μg/ml poly(dI)poly(dC), and the bound proteins were solubilized in sample buffer, boiled, and subjected to SDS-PAGE. Membranes were probed with antibodies against p65, p50, CREB, c-Jun, TBP, HMG-I(Y), CBP, or NF-Y at dilutions ranging from 1:2,500 to 1:10,000 followed by chemiluminescent detection.

**In Vivo Phosphorylation of p65 and TFIIID (TBP)—Confluent monolayers of THP-1 cells in 10-cm tissue culture dishes were labeled with 200 μCi of 32P-orthophosphate (400–800 μCi/ml; Perkin-Elmer Life Sciences) in phosphate-free RPMI medium with 10% fetal calf serum for 3 h at 37 °C. Cells were stimulated with LPS in the absence or presence of VIP or PACAP for 1 h, washed, and resuspended in lysis buffer (1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 10 mM Na3PO4 (pH 7.2), 2 mM EDTA, 50 mM NaF, 0.2 mM Na3VO4, 1 μM okadaic acid, 100 μg/ml phenylmethylsulfonyl fluoride, 50 μg/ml aprotinin, 10 μg/ml leupeptin, 50 μg/ml pepstatin), and sonicated. The p65 or TFIIID (TBP) were immunoprecipitated with rabbit anti-p65 or anti-TFIIID (TBP) antibodies (Santa Cruz Biotechnology) and bound to Gammabind (Amersham Pharmacia Biotech) for 2–12 h at 4 °C. The pellets were washed twice with high salt, followed by electrophoresis on 8–10% SDS-PAGE gels and autoradiography.

**ELISA—TNFα levels in culture supernatants** were determined using a human-specific sandwich ELISA (PharMingen) following the manufacturer's instructions.

**RT-PCR for the Detection of VPAC1, VPAC2, and PAC1 mRNA Expression**—THP-1 cells were cultured at a concentration of 2 × 106 cells/ml in 100-mm tissue culture dishes and stimulated with LPS (0.5 μg/ml) for up to 12 h. Two μg of total RNA was reverse-transcribed, and cDNA was amplified with specific primers. Glyceraldehyde-3-phosphate dehydrogenase primers (Stratagene) were used as control. The primers for VPAC1, VPAC2, and PAC1 receptors have been described before (25).

**RESULTS**

**VIP and PACAP Inhibit LPS-induced TNFα Production in Human Monocytes**—We have recently demonstrated that VIP and PACAP inhibit TNFα production in murine peritoneal macrophages and the macrophage cell line Raw 264.7 (19, 25). To investigate the effects of VIP/PACAP on human monocytes, THP-1 cells were stimulated with LPS in the presence or absence of VIP or PACAP, and the amounts of secreted TNFα were determined by ELISA. VIP and PACAP inhibit TNFα production in a dose-dependent manner, with a maximum effect in the concentration range of 10−5 to 10−6 M (Fig. 1A). Both neuropeptides inhibit in a time-dependent manner TNFα steady-state mRNA levels (Fig. 1B). MG132, a newly described NF-κB inhibitor, shows a similar inhibitory effect on TNFα mRNA (Fig. 1B).

**VIP and PACAP Inhibit LPS- and TNFα-induced NF-κB-dependent Transcription in Monocytes**—Since LPS up-regulates TNFα transcription through an NF-κB-dependent mechanism (31, 32), we determined whether VIP and PACAP inhibit NF-κB transcriptional activity. THP-1 cells were transiently transfected with the (κB)4-luciferase reporter plasmid, containing four copies of the NF-κB consensus site. Forty eight hours later, the cells were stimulated with LPS or TNFα in either the presence or absence of VIP or PACAP and were assayed for NF-κB-dependent transcription 5 h later. Both LPS and TNFα led to an ∼18-fold increase in NF-κB transcriptional activity (Fig. 2A). Treatment with VIP or PACAP strongly inhibits LPS- or TNFα-induced NF-κB activity (Fig. 2A). The inhibitory effect is dose-dependent (Fig. 2B).

**VIP and PACAP Inhibit NF-κB Nuclear Translocation by Preventing LPS-induced Phosphorylation/Degradation of IκBα**—To assess whether VIP and PACAP inhibit NF-κB DNA binding, EMSAs were performed. Stimulation of THP-1 cells with LPS led to strong NF-κB binding showing a maximum increase at 2 h after stimulation, and VIP and PACAP inhibit binding at all time points (Fig. 3A). The binding specificity was ascertained by the displacement with 50-fold excess of unlabeled homologous (NF-κB) but not nonhomologous oligonucleotide (CREB) (Fig. 3A). Antibody supershift experiments indicate the presence of both p50 and p65, with no supershift for an irrelevant Ab (anti-CREB) (Fig. 3A).

The primary level of control for NF-κB is mediated through its interaction with the inhibitor IκB. VIP and PACAP could inhibit NF-κB activity by blocking LPS-induced IκB degradation and subsequent NF-κB nuclear translocation. We measured the levels of p65 in cytoplasm and nucleus. As expected, p65 was predominantly localized in the cytoplasm of unstimulated cells, and LPS induced a decrease in the level of cytoplasmic p65, and an increase in nuclear p65 levels (Fig. 3B). VIP and PACAP abolished the LPS-induced change in p65 levels (Fig. 3B), which indicates an inhibition of p65 nuclear translo-
cation. Similar levels of p50 indicate equal protein loading. To
determine whether VIP/PACAP interfere with the LPS-in-
duced degradation of I\(\kappa\)B, we examined cytoplasmic I\(\kappa\)B levels. As expected, we observed a time-dependent I\(\kappa\)B degradation, paralleled by an increase in I\(\kappa\)B phosphorylation in LPS-stimulated cells (Fig. 3C). VIP and PACAP block the phos-
phorylation and subsequent degradation of I\(\kappa\)B (Fig. 3C). In
addition, we determined the effect of VIP and PACAP on I\(\kappa\)B degradation by transiently transfecting an EGFP-tagged I\(\kappa\)B construct in THP-1 cells and assaying the percentage of fluo-
rescent I\(\kappa\)B \(^1\) cells at different time points by flow cytometry.
LPS led to a rapid decrease in I\(\kappa\)B \(^1\) cells, whereas VIP signif-
ically increased I\(\kappa\)B half-life in LPS-treated cells (Fig. 3C, right panel).

**FIG. 2.** VIP and PACAP inhibit LPS- and TNFα-induced NF-κB transcrip-
tional activity. THP-1 cells were trans-
fected with the (κB)\(_4\)-Luc construct (10 μg) and treated 48 h later with LPS or
TNFα (10 ng/ml), with or without VIP or PACAP (10^{-8} M for A) for 6 h. Cytosolic
extracts (100 μg) were used in luciferase assays. Fold induction is relative to lucif-
erase activity in unstimulated cells. Data
are expressed as the mean ± S.D. of three
independent experiments performed in
duplicate.

**FIG. 3.** VIP and PACAP prevent LPS-induced IκB degradation and the subse-
quent NF-κB nuclear translocation. **A,** VIP and PACAP inhibit NF-κB DNA binding. Nuclear extracts from cells incubated for 2 h with LPS with or without VIP or PACAP (10^{-8} M) were used in EMSA. **Probe,** consensus NF-κB. **Supershift,** nuclear extracts were incubated with polyclonal antibodies against p65, p50, or CREB for 20 min before the
addition of the κB probe. One representative experiment of three is presented. **Right panel,** NF-κB binding at various time points expressed as
arbitrary densitometric units. Data represent the mean ± S.D. of three independent assays. **B,** VIP and PACAP inhibit p65 translocation. Cells
were treated with LPS with or without VIP or PACAP (10^{-8} M) for 1 h. Cytosolic and nuclear proteins were extracted, and Western blot analysis
was performed for p50 and p65. One representative experiment of three is shown. **C,** VIP and PACAP prevent IκB phosphorylation and
degradation. **Left panels,** THP-1 cells were stimulated with LPS with or without VIP or PACAP (10^{-8} M). The cytosolic amounts of IκBα and
phosphorylated IκBα at different time points were determined by Western blot. One representative experiment of three is shown. **Right panels,**
THP-1 cells were transiently transfected with a fluorescent IκB-EGFP signaling probe and treated with LPS with or without 10^{-8} M VIP 24 h later. The percentage of IκB^- cells was determined by flow cytometry at different time points. Similar results were obtained in three independent experiments. **D,** VIP and PACAP inhibit IKKα activity. THP-1 cells were stimulated with LPS with or without VIP or PACAP (10^{-8} M) (10 min in
**upper panel**). IKKα activity was assayed in an in vitro kinase assay. **Lower panel,** IKKα activity is expressed as arbitrary densitometric units. Data
represent the mean ± S.D. of three independent assays. IKKα protein amounts were determined by immunoblotting (**upper panel**; control). **E,**
overexpression of p65 partially reverses the inhibitory effect of VIP and PACAP. THP-1 cells were transiently cotransfected with the (κB)\(_4\)-Luc
construct (10 μg) and increasing concentrations (0, 2.5, 5, 10, or 15 μg) of pRSV-p65 vector (p65). The cells were stimulated 48 h later with LPS
with or without 10^{-8} M VIP or PACAP and incubated for 6 h before determining luciferase activity. Fold induction is relative to luciferase activity in
unstimulated cells. Results represent the mean ± S.D. of three independent experiments performed in duplicate.
Since the LPS activation of NF-κB requires IKK-mediated phosphorylation of IκBα (1, 2), we determined if VIP and PACAP inhibit IKK activity by using an in vitro kinase assay. Stimulation of THP-1 cells with LPS resulted in a time-dependent increase in IKKα activity, which was inhibited by VIP and PACAP (Fig. 3D). No differences in IKKα expression were observed (Fig. 3D).

These results demonstrate that VIP and PACAP inhibit NF-κB nuclear translocation and subsequent DNA binding in LPS-stimulated cells by blocking the IKK-mediated IκB phosphorylation/degradation. If the inhibitory effect of VIP and PACAP on NF-κB transcriptional activity is mediated entirely through the inhibition of NF-κB nuclear translocation, overexpression of p65 should reverse this effect. Therefore, THP-1 cells were transiently cotransfected with the (κB)2-luciferase reporter plasmid and increasing concentrations of a vector expressing p65. Increasing concentrations of p65 only partially reversed the inhibitory effect of VIP and PACAP, suggesting that the neuropeptides affect more than NF-κB nuclear translocation.

**Neither VIP nor PACAP Affect LPS-induced p65 Phosphorylation.**—Several studies have demonstrated that p65 is phosphorylated during in vitro NF-κB activation, leading to increased transcriptional activity (10, 33). We examined the effects of VIP and PACAP on the phosphorylation of transiently transfected p65. VIP and PACAP had no effect on LPS-induced p65 phosphorylation (Fig. 4, upper panel). Similar levels of p65 were detected by Western blotting in THP-1 cells transfected with p65 in the presence or absence of LPS, VIP, or PACAP (Fig. 4, lower panel).

**VIP and PACAP Promote CREB/CBP Versus p65/CBP Interactions by Increasing CREB Phosphorylation/Activation.**—In addition to DNA binding, the interaction of p65 with CBP is essential for optimal NF-κB transcriptional activity (9). In addition to p65, CBP interacts with other factors including CREB (6). Changes in p65 phosphorylation or competition with other factors for the limiting quantities of nuclear CBP lead to changes in p65/CBP interactions. THP-1 cells were stimulated with LPS in the absence or presence of VIP or PACAP, and total cell lysates were immunoprecipitated with antibodies to p65 or CREB and probed for the presence of CBP. LPS stimulation results in the appearance of p65/CBP complexes (Fig. 5A). No p65-CBP complexes are detected in unstimulated cells. VIP and PACAP decrease the levels of p65/CBP and increase the levels of CREB-CBP complexes (Fig. 5A). Moreover, VIP and PACAP induce CREB-CBP instead of p65-CBP complexes even in the presence of overexpressed CBP (Fig. 5A).

To confirm that the VIP/PACAP inhibition of NF-κB transcriptional activity is related to the decrease in p65/CBP complexes, THP-1 cells were cotransfected with the (κB)2-luciferase reporter system and increasing concentrations of p65 and/or CBP. Expression of increasing concentrations of CBP led to a partial reversal of the inhibitory effect of VIP/PACAP on NF-κB activation (Fig. 5B, upper panel). A similar conclusion was reached earlier regarding p65 (Fig. 3E). However, the coexpression of CBP (fixed concentration) and p65 (increasing concentrations) completely reversed the VIP/PACAP effect (Fig. 5B, upper panel). This correlates with the fact that coexpression of p65 and CBP restored the p65-CBP complexes to levels observed in the LPS-treated cells (Fig. 5B, lower panel).

The fact that, even in the presence of excess p65, the levels of p65-CBP complexes and the NF-κB transcriptional activity are not completely restored (Figs. 5B and 3F) indicates that VIP and PACAP affect the formation of p65-CBP complexes through both a reduction in nuclear p65 and a direct effect on CBP.

Since VIP receptors are mostly linked to the cAMP/PKA pathway, it is highly possible that VIP and PACAP activate CREB which then recruits CBP. Therefore, we analyzed the effects of VIP and PACAP on CREB phosphorylation. LPS increases CREB phosphorylation slightly as compared with unstimulated controls (Fig. 5C, upper panels). In contrast, VIP and PACAP strongly augment the levels of phosphorylated CREB (Fig. 5C, upper panels). Total CREB levels were not affected by either treatment. In addition, CREB levels in cytoplasmic, and nuclear extracts were assayed by Western blotting. LPS stimulation results in a slight increase in nuclear CREB, and treatment with VIP or PACAP leads to high levels of nuclear CREB (Fig. 5C, lower panels).

To determine whether VIP/PACAP-induced CREB phosphorylation correlates with increased DNA binding and CREB-dependent transcription, we performed EMSAs using a consensus CRE site and transient transfections with a CRE-luciferase reporter plasmid. LPS leads to a slight increase in CRE DNA binding, and VIP and PACAP strongly augment this binding (Fig. 5D). The binding specificity was confirmed by using homologous (CRE) and nonhomologous (NF-κB) oligonucleotides as competitors (Fig. 5D). The CRE complexes are supershifted by an anti-CRE Ab but not by an anti-p65 Ab (Fig. 5D). In cells transfected with CRE-luciferase constructs, VIP and PACAP significantly increase the CRE-dependent transcriptional activity, as compared with cells treated with LPS alone (Fig. 5D). These results indicate that VIP and PACAP increase the phosphorylation/activation of CREB which then competes with p65 for limiting amounts of CBP, resulting in increased CREB-CBP and decreased p65-CBP complexes.

**VIP and PACAP Reduce LPS-induced TBP DNA Binding Activity and Its Interaction with p65 by Inhibiting the MEKK1-MEK3/6-p38 MAPK Pathway.**—Since NF-κB-driven transcription also depends on the activation of basal transcription factors, such as TFIIB and TFIIID (TBP), we investigated if VIP and PACAP regulate the basal transcriptional factors. We determined first the effect of VIP and PACAP on TBP binding to the TATA box. LPS increases TBP binding, and VIP and PACAP reduce it to control levels. The specificity of TBP binding is indicated by competition of 50-fold excess of unlabeled...
homologous (TBP), but not nonhomologous, oligonucleotide (NF-AT). The TBP complexes are supershifted by an anti-TBP Ab but not by an irrelevant Ab (anti-CREB) (Fig. 6A).

Since the association of the carboxyl terminus of p65 with TBP and TBP is known to be important for the transcriptional regulation of NF-κB (34, 35), we determined whether VIP and PACAP regulate p65/TBP interactions by immunoprecipitating cell lysates with anti-p65 Abs and immunoblotting with TBP. LPS increases this interaction, and VIP and PACAP inhibit the LPS-induced p65/TBP interaction (Fig. 6B, upper panel). To determine whether the effect of VIP/PACAP is due to the previously described inhibition of p65 nuclear translocation, THP-1 cells were transiently transfected with increasing concentrations of p65. Overexpression of p65 partially reversed the effect of VIP (Fig. 6B, lower panel, compare 2nd to 5th lanes to the 6th lane). However, the incomplete reversal suggests that VIP and PACAP might directly regulate TBP activation.

Since TBP is activated following phosphorylation by the p38 MAP kinase, we evaluated the effect of VIP and PACAP on TBP phosphorylation. Whereas no TBP phosphorylation is observed in unstimulated cells, LPS induces high levels of phosphorylated TBP (Fig. 6C). VIP and PACAP inhibit TBP phosphoryl-

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**FIG. 5.** VIP and PACAP promote CREB/CBP versus p65/CBP interactions by increasing CREB phosphorylation. A, VIP and PACAP promote CBP/CREB and inhibit CBP-p65 complex formation. Upper panels, THP-1 cells were treated with LPS with or without VIP or PACAP (10^{-8} M) for 1 h. Cell extracts were subjected to immunoprecipitation (IP) with anti-CREB or anti-p65 antibodies and analyzed by Western blot with anti-CBP. Data are representative of four experiments. Lower panels, THP-1 cells were transiently transfected with the pRSV-CBP vector containing CBP cDNA coupled to HA tag. After 24 h, the cells were treated with LPS with or without VIP or PACAP (10^{-8} M) for 1 h. Cell extracts were subjected to immunoprecipitation (IP) with anti-CREB or anti-p65 antibodies and analyzed by Western blot with anti-HA. Data are representative of three experiments. **B**, effects of CBP and CBP plus p65 overexpression. Upper panel, THP-1 cells were cotransfected with the (κB)_2-Luc construct (10 μg) and increasing concentrations (0 μg for 1st and 2nd lanes, and 2.5, 5, 10, and 15 μg for 3rd to 6th lanes, and 5 μg for 7th to 10th lanes) of pRSV-CBP vector (CBP) and increasing concentrations (0 μg for 1st to 6th lanes and 2.5, 5, 10, or 15 μg for 7th to 10th lanes) of pRSV-p65. After 48 h, cells were stimulated with LPS in the absence (1st lane) or presence of 10^{-8} M VIP (2nd to 10th lanes) and incubated for an additional 5 h before determining luciferase activity. Fold induction is relative to luciferase activity in unstimulated cells. Results represent the mean ± S.D. of four experiments. **C**, CREB activation. Upper panel, THP-1 cells were treated with LPS with or without VIP or PACAP (10^{-8} M) for 1 h. Upper panels, cell extracts were analyzed by Western blot using anti-phosphorylated CREB or anti-CREB. Data are representative of four experiments. Lower panels, cytosolic and nuclear proteins were extracted and subjected to Western blot with anti-CREB. Data are representative of three experiments. **D**, CRE binding and activation. Upper panel, nuclear extracts from cells incubated for 2 h with LPS with or without VIP or PACAP (10^{-8} M) were assayed for DNA binding by EMSA. Probe, the consensus CRE site. Supershift, nuclear extracts were incubated with polyclonal antibodies against p65 or CREB for 20 min before the addition of the CRE probe. Similar results were observed in other three experiments. Right panel, THP-1 cells were transfected with the CRE-Luc construct (10 μg). After 48 h cells were treated with LPS with or without VIP or PACAP (10^{-8} M) for 5 h. Cytosolic extracts (100 μg) were used in the luciferase assay. Data are expressed as the mean ± S.D. of relative luciferase units (RLU) from three independent experiments performed in duplicate.
subjected to immunoprecipitation (IP) labeled with 32Pi in phosphate-free media for 3 h. Cells were treated in vivo and PACAP inhibit with anti-TBP. Data are representative of four experiments.

The mean activity is expressed as arbitrary densitometric units. Data represent p38 MAPK, MEK3, MEK6, and MEKK1 were determined by immunoblotting.

VIP and PACAP Change the LPS-induced Composition of Nuclear Factors Bound to TNFα Promoter in Human Monocytic Cells—We next investigated the effects of VIP and PACAP on the LPS-induced transcriptional activators binding the proximal regulatory region in the human TNFα promoter, which contains two essential transactivating binding sites, i.e. the NF-κB and CRE elements. Depending on the activation state, the CRE site may bind either CREB or c-Jun, with CREB preferentially bound in unstimulated cells and c-Jun in LPS-stimulated cells. To determine whether the activators present in nuclear extracts would bind to the TNFα promoter, a biotinylated affinity matrix spanning the proximal regulatory region of human TNFα promoter was generated and coupled to streptavidin-coated magnetic beads. This biotinylated probe was incubated with nuclear extracts from unstimulated or LPS-stimulated THP-1 cells treated with or without VIP or PACAP. Transcription factor complexes were released from the magnetic beads by boiling in SDS sample buffer and detected by immunoblotting.

p65 is present in LPS-treated samples, but not in unstimulated or stimulated cells treated with VIP or PACAP (Fig. 7, p65, input). The p65 present in the LPS-treated cells binds to the TNFα promoter region (Fig. 7, p65, bound). In contrast, p50 is constitutively expressed in THP-1 cells and binds partially to the TNFα promoter (Fig. 7, p50, input, bound, and flow-thru). The p50 binding is not affected by LPS, VIP, or PACAP (Fig. 7, p50). CREB is slightly induced by LPS and highly induced by VIP and PACAP (Fig. 7, CREB, input). All induced CREB binds to the TNFα promoter region (Fig. 7, CREB, bound, and flow-thru). Both TBP and CBP are constitutively present in the nucleus, and neither LPS nor VIP/PACAP affect their levels (Fig. 7, CBP and TBP, input). However, whereas CBP from unstimulated, LPS-stimulated, and VIP/PACAP-treated cells is all bound to the TNFα promoter region, binding of TBP is induced by LPS and inhibited by VIP and PACAP (Fig. 7, CBP and TBP, bound). Similar to TBP, c-Jun is present in the nucleus and binds to the TNFα promoter region when the cells are stimulated with LPS, and this binding is inhibited by VIP and PACAP (Fig. 7, c-Jun). As a control, we used the nuclear factor-Y (NF-Y), a transcription factor present in the nucleus which binds constitutively to various promoters, including TNFα. Both VIP and PACAP significantly reduce the phosphorylation of all these kinases (Fig. 6D). None of these treatments affected the expression of any of the kinases assayed (Fig. 6D). We conclude that the VIP/PACAP inhibition of TBP phosphorylation is mediated through the inhibition of the MEKK1/MEK3/MEK6/p38 MAPK cascade.

VIP and PACAP Inhibit NF-κB Transactivating Activity 375
activation, i.e. the nonhistone chromosomal proteins of the high mobility group (HMG-I(Y) family, two chromatin architectural proteins that play a role in the transcriptional regulation of certain mammalian genes (39, 40). HMG-I(Y) was shown to enhance the DNA binding of several transcription factors, including NF-κB (41, 42). HMG-I(Y) was present in the nucleus from unstimulated, LPS-stimulated, and VIP/PACAP-treated THP-1 cells, and none of the treatments affected its binding to the TNFα promoter, suggesting that HMG-I(Y) is an unlikely element in the regulation of NF-κB activation by VIP and PACAP.

**Involvement of VPAC1 and cAMP/PKA in the Effects of VIP and PACAP on NF-κB-mediated Gene Activation**—Although THP-1 cells were previously shown to express VIP/PACAP-binding sites, primarily coupled to cAMP production (43), the nature of these binding sites was not elucidated. We investigated the expression of VPAC1, VPAC2, and PAC1 by RT-PCR in unstimulated and LPS-stimulated THP-1 cells. Both VPAC1- and PAC1-specific fragments were amplified from unstimulated and LPS-stimulated THP-1 cells. Both VPAC1- and PAC1-specific fragments were amplified from unstimulated, LPS-stimulated, and VIP/PACAP-treated THP-1 cells. Both VPAC1- and PAC1-specific fragments were amplified from unstimulated and LPS-stimulated monocytes, whereas VPAC2 fragments were only detected in stimulated cells (Fig. 8A).

Our previous studies identified VPAC1 and cAMP as the major mediators of VIP/PACAP effects on macrophage-derived cytokines (18–21, 22–27). However, VIP and PACAP inhibit NF-κB nuclear translocation in murine macrophages through a cAMP-independent pathway (18, 25–27). To determine the receptor and the transduction pathways involved, we used specific VPAC antagonists and a specific protein kinase A inhibitor (H89). The VIP inhibition of NF-κB transcripional activity is completely reversed by the VPAC1 antagonist and only slightly reversed by increasing concentrations of H89. In addition, forskolin (a cAMP-inducing agent) mimics only partially the effect of VIP (Fig. 8B). These findings suggest that whereas the effect of VIP on NF-κB activation is entirely VPAC1-dependent, it is mediated by both a cAMP-dependent and a cAMP-independent pathway. When NF-κB DNA binding and IκB phosphorylation were analyzed, we found again that the VPAC1 antagonist completely reversed the inhibitory effect of VIP and that H89, even at the highest concentrations used, only minimally reversed this effect. This correlates with the fact that forskolin inhibits only weakly the NF-κB DNA binding and IκB phosphorylation (Fig. 8C). Therefore, the major pathway for the inhibition of NF-κB nuclear translocation by VIP is non-cAMP mediated.

In contrast, the VPAC1 antagonist and the PKA inhibitor completely reversed the effect of VIP on CREB phosphorylation, and forskolin entirely mimicked the effect of VIP (Fig. 8A). A similar conclusion was reached for the effect of VIP on the preferential induction of CREB-CBP versus p65-CBP complexes (Fig. 8A). Finally, the VPAC1 antagonist and H89 also reversed the effects of VIP on the phosphorylation of TBP and the activation of the MEKK1/MEK3/p38 MAPK pathway, with forskolin mimicking the effects of VIP (Fig. 9B). These results suggest that the regulatory activities of VIP on CBP and TBP are mediated entirely through the cAMP/PKA pathway.

**DISCUSSION**

VIP and PACAP control inflammatory processes by suppressing the monocyte/macrophage production of several proinflammatory factors known to be transcriptionally controlled by NF-κB (21–32). Although VIP and PACAP have been found to inhibit the translocation of NF-κB and its DNA binding in mouse macrophages (18, 25–27), the effects of these neuropeptides on NF-κB-dependent transcriptional activity and the detailed molecular mechanisms governing this process have still to be elucidated. The present study shows that VIP and PACAP specifically inhibit the LPS-induced NF-κB transcriptional activity in the human monocytic cell line THP-1.

VIP and PACAP operate at three different levels to inhibit the NF-κB transcriptional activity. First, VIP and PACAP inhibit p65 nuclear translocation and subsequent DNA binding. This process is mediated through the VPAC1 receptor and a non-cAMP transduction pathway. Second, VIP and PACAP induce CREB phosphorylation, and the phosphorylated CREB competes with p65 for the coactivator CBP. Third, VIP and PACAP inhibit the MEKK1/MEK3/p38 pathway ultimately affecting the phosphorylation of TBP and its binding to both p65 and the TNFα promoter. The effects on CBP and TBP are both mediated through VPAC1 and the cAMP/PKA transduction pathway.

The inhibition of p65 translocation by VIP/PACAP is mediated through the stabilization of IκBα. IκBα, a member of the
IκB family, is the major player in the response to inflammatory stimuli. Upon phosphorylation at specific serine residues by the kinases IKKα and -β, IκBα is ubiquitinated and degraded by the 26 S proteasome (reviewed in Ref. 2). As previously demonstrated for murine macrophages and T cells (27), VIP and PACAP inhibit IκBα phosphorylation and its subsequent degradation. This is accomplished through an inhibitory effect on IKKα. Similar results were obtained in this study. The inhibitory effect of VIP and PACAP on p65 nuclear translocation and NF-κB DNA binding has functional consequences, since overexpression of p65 partially reverses this inhibitory effect. The fact that the reversal is incomplete suggests additional mechanisms for the inhibition of NF-κB transcriptional activity. To investigate this hypothesis several possibilities were considered.

It has been reported that the catalytic subunit of PKA associates with the cytosolic NF-κB-IκB complex phosphorylating p65 and that p65-mediated transcription is strongly dependent on its phosphorylation (10). However, our results clearly demonstrate that neither VIP nor PACAP affect the in vivo LPS-induced phosphorylation of p65.

An additional regulatory element in the NF-κB transcriptional activity is the coactivator CBP. CBP performs an important role in the integration of diverse signaling pathways by...
linking p65 with components of the basal transcriptional machinery, such as TFIIB, TBP, and histone acetyltransferases (44). The present report demonstrates that VIP and PACAP indeed inhibit the formation of p65-CBP complexes and that this event is directly related to the inhibition of NF-κB transcriptional activity. The fact that overexpression of p65 did not completely reverse the VIP/PACAP inhibition of p65-CBP complex formation suggests that VIP/PACAP might directly affect CBP. Since CBP is in limiting amounts in the nucleus and is capable to interact with several transcriptional factors (3, 6–13), competition for CBP provides another mechanism for transcriptional regulation (8, 45, 46). CBP binds to phosphorylated CREB, and formation of CREB-CBP complexes reduces the CBP available for complexing with p65 (8, 9). VIP/PACAP were shown to induce CREB DNA binding in activated murine macrophages (25, 26). This study shows that VIP and PACAP increase CBP binding to CREB, replacing p65-CBP with CREB-CBP complexes in LPS-stimulated THP-1 cells. This is due to VIP/PACAP-induced increases in CREB phosphorylation/activation. The fact that cotransfections with p65 and CBP completely reverse the inhibitory effect of VIP/PACAP on NF-κB transcriptional activity, whereas p65 and CBP separately result in only a partial reversal, suggests that VIP/PACAP operate by inhibiting both p65 nuclear translocation and CBP availability.

The observation that VIP and PACAP induce high levels of nuclear CREB was unexpected. In most systems, CREB is localized in the nucleus in both stimulated and unstimulated cells and becomes transcriptionally active upon phosphorylation (reviewed in Ref. 47). However, in our system, the unstimulated THP-1 cells express mostly cytoplasmic CREB, and VIP/PACAP induce significant levels of nuclear CREB. This might be a characteristic of this particular cell line, with VIP/PACAP contributing to the retention of phosphorylated CREB in the nucleus.

In addition, p65 was shown to interact with TBP and TFIIB of the basal transcriptional complex, and these interactions appear essential for optimal NF-κB-driven transcription (48). The interaction of p65 with TBP and TFIIB is presumably mediated through CBP (reviewed in Ref. 4). Our study indicates that VIP and PACAP inhibit LPS-induced TBP binding to the TATA box and p65/TBP interaction. Similar to CBP, TBP is found in limiting amounts in the nucleus but is activated following LPS stimulation by the MEKK1/MEK3/MEK6/p38

**Fig. 10.** Model for the inhibitory effect of VIP and PACAP on LPS-induced NF-κB-dependent gene activation (see “Discussion” for details).
VIP and PACAP Inhibit NF-κB Transactivating Activity

MAPK pathway (36–38, 50). Several studies demonstrated the involvement of p38 MAPK in the activation of NF-κB (50–52). VIP and PACAP inhibit the p38 MAPK pathway and the subsequent TBP phosphorylation/activation.

Finally, we investigated another possible regulatory element in this system. HMG-I and HMG-Y are two architectural proteins that facilitate the assembly of functional nucleoprotein complexes by modifying DNA conformation and recruiting nuclear proteins to the promoter. HMG-I(Y) also enhance the DNA binding of NF-κB (41). VIP and PACAP did not affect either the nuclear expression or the DNA binding of HMG-I(Y) in LPS-stimulated THP-1 cells.

VIP and PACAP act through three specific receptors, i.e. VPAC1, VPAC2, and PAC1 (53). Human monocytes, constitutively, and VPAC2 following LPS stimulation. Similarly, in the present study we have found that VIP and PACAP regulate NF-κB transcriptional activity through both cAMP-dependent and -independent pathways. VIP/PACAP regulation of both the MEKK1/MEK3/p38 MAPK-mediated TBP activation and CREB/CBP interactions is entirely cAMP-dependent. CREB phosphorylation was previously shown to be mediated by the cAMP/PKA pathway (reviewed in Ref. 47). On the other hand, the neuropeptide-mediated inhibition of IκB phosphorylation and p65 nuclear translocation is mainly cAMP-independent. This is in agreement with previous reports showing that the nuclear expression or the DNA binding of HMG-I(Y) to multiple sites increases the binding affinity of NF-κB and binds DNA to facilitate the formation of a higher order transcriptional complex. These events place multiple transcriptional activators in a favorable position to compete for the coactivator CBP present in limiting amounts. CBP acts as an efficient integrator, bridging transactivators to the components of the basal machinery TFIIB and TBP. Since RNA polymerase II is constitutively associated with CBP, binding of the coactivator to the promoter facilitates the recruitment of the polymerase. VIP and PACAP dramatically change this optimal transcriptional conformation. Binding of VIP or PACAP to VPAC1 initiates two transduction pathways. The cAMP-dependent pathway leads to the phosphorylation/activation of CREB, which binds to the CRE site in the promoter and competes with p65 for limiting amounts of CBP. The cAMP-dependent pathway also inhibits MEKK1 activity, resulting in the inhibition of both c-Jun and TBP phosphorylation/activation. On the other hand, the cAMP-independent pathway inhibits the IKK-mediated IκB phosphorylation and subsequent NF-κB nuclear translocation and DNA binding. A link between the two pathways could be established through the regulation of IKK activity by MEKK1 (49, 55). As a result of the activation of the two pathways, VIP and PACAP block NF-κB transcriptional activity.

Since NF-κB positively regulates the transcription of different monocye/macrophage-derived proinflammatory genes, which are commonly associated with inflammatory and autoimmune disorders, the inhibition of the NF-κB transcriptional activity by VIP and PACAP could have significant therapeutic potential. The fact that VIP and PACAP regulate NF-κB activation at multiple levels, and through different transduction pathways, could offer a significant advantage over other anti-inflammatory agents.

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