



Vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide inhibit CBP–NF- κ B interaction in activated microglia

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Abstract

The vasoactive intestinal peptide (VIP) and the pituitary adenylate cyclase-activating polypeptide (PACAP), two immunomodulatory neuropeptides, act as anti-inflammatory factors for activated microglia, by inhibiting the production of pro-inflammatory factors, mainly mediated through the inhibition of NF- κ B nuclear translocation and DNA binding. An additional regulatory element in the NF- κ B transcriptional activity is the coactivator CBP, which links p65 with components of the basal transcriptional machinery. The present report demonstrates that VIP and PACAP inhibit the formation of p65/CBP complexes and that this event is directly related to the neuropeptide inhibition of NF- κ B transcriptional activity. Since CBP is in limiting amounts in the nucleus and is capable of interacting with several transcriptional factors, competition for CBP provides another mechanism for transcriptional regulation. VIP and PACAP increase CBP-binding to CREB, replacing p65/CBP with CREB/CBP complexes in activated microglia. This is due to VIP/PACAP-induced increases in CREB phosphorylation/activation and is mediated through the specific VPAC1 receptor and the cAMP/PKA pathway. The VIP/PACAP interference with the p65/CBP interaction in activated microglia may represent a significant element in the regulation of the inflammatory response in the CNS by the endogenous neuropeptides.

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Despite its central role in the regulation of immune and inflammatory activities, as well as tissue remodeling in the CNS, activated microglia may also play a pathogenetic role in a variety of CNS diseases, involving the secretion of microglia-derived pro-inflammatory mediators, including TNF α , IL-1 β , IL-6, IL-12, and nitric oxide (NO) [1].

The pleiotropic transcription factor NF- κ B plays an important role in the transcriptional regulation of all these genes (reviewed in [2]). NF- κ B occurs in both homo- and heterodimeric forms. The most common transcriptionally active form is a p50/p65 heterodimer (reviewed in [3]). In unstimulated cells, NF- κ B is localized in the cytosol bound to inhibitor proteins, collectively termed I κ B. Cellular stimulation with

inflammatory cytokines, phorbol esters, UV irradiation, or potent oxidants results in I κ B phosphorylation, ubiquitination, and proteosomal degradation [2,3]. This is followed by the rapid translocation of NF- κ B to the nucleus where it binds to specific κ B elements within promoters [2].

Several studies have shown that the transactivating activity of NF- κ B 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 [4]. 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 [5]. CBP functions as an integrator linking various transcription factors to the basal transcriptional apparatus,

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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 [6–8]. The interaction of p65 with CBP is essential for NF- κ B transcriptional activity [9–11] and this interaction can be strengthened by p65 phosphorylation [6,12], or impeded by competition from other CBP-binding factors such as CREB, c-Jun, c-Fos, p53, steroid receptors, c-Myb, and Myo-D [8,13–17].

Vasoactive intestinal peptide (VIP) and the structurally related peptide, the pituitary adenylate cyclase-activating polypeptide (PACAP), are two neuropeptides that elicit a broad spectrum of biological functions, including actions on natural and acquired immunity [18], although their primary immunomodulatory function is anti-inflammatory in nature. VIP and PACAP inhibit cytokine production and proliferation in T cells, and several macrophage functions, including phagocytosis, respiratory burst, and chemotaxis [18], as well as LPS-induced IL-6, TNF α , IL-12, NO, and chemokine production [19–23]. Similarly, we have recently demonstrated that both neuropeptides act as two potent microglia-deactivating factors by inhibiting the production of LPS-induced pro-inflammatory mediators [24,25]. The inhibition of pro-inflammatory mediators is responsible, at least partially, for the protective effect of VIP and PACAP in vivo in a murine model for septic shock [26], and might contribute to their role as survival factors for neuronal cells in injury models ([25,27]). Many of the pro-inflammatory cytokines affected by VIP and PACAP are known to be regulated by NF- κ B [2]. In fact, we have previously demonstrated that VIP and PACAP inhibit NF- κ B nuclear translocation and DNA binding to several promoters in activated microglia [24,25]. However, the possibility that VIP and PACAP could regulate NF- κ B transcriptional activity in activated microglia through the regulation of coactivators has not been investigated to date. The present study shows that VIP and PACAP selectively inhibit the interaction of p65 with CBP, while increasing interactions between CBP and CREB, by increasing the phosphorylation/activation of CREB. The VIP/PACAP effect is mediated through the VIP/PACAP receptor VPAC1 and is cAMP-dependent.

Materials and methods

Reagents. Synthetic VIP and PACAP38 were purchased from Novabiochem (Laufelfingen, Switzerland). The VPAC1-antagonist [Ac-His¹, D-Phe², K¹⁵, R¹⁶, L²⁷] VIP [3–7]-GRF [8–27] was donated by Dr. Patrick Robberecht (Universite Libre de Bruxelles, Belgium). The PAC1-antagonist PACAP_{6–38} was obtained from Peninsula Laboratories (Belmont, CA). Mouse recombinant TNF α was purchased from Pharmingen (San Diego, CA). Lipopolysaccharide (LPS from *Escherichia coli* 055:B5) and forskolin were purchased from Sigma Chemicals (St. Louis, MO), and H89 was from ICN Pharmaceuticals

(Costa Mesa, CA). Antibodies against c-Fos, CREB, and p65 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody against phosphorylated CREB was purchased from New England Bio Labs (Beverly, MA).

Cell cultures. Microglial cell cultures were prepared as previously described [28]. Purified microglial cell cultures were composed of a cell population in which >98% stained positively with MAC-1 antibodies (Boehringer–Mannheim Biochemicals, Indianapolis, IN) and <2% stained positively with antibodies specific to the astrocyte marker glial fibrillary acid protein (Sigma). Microglia monolayers were stimulated with LPS (1 μ g/ml) or TNF α (20 ng/ml) in the presence or absence of VIP or PACAP38 (10^{−8} M) for various times at 37 °C in a humidified incubator with 5% CO₂.

Immunoprecipitation experiments and Western blotting. Cell lysates were prepared by lysing 5 × 10⁶ cells in lysis buffer (20 mM Hepes, pH 7.4, 150 mM NaCl, 2 mM EGTA, 50 mM glycerolphosphate, 1% Triton X-100, 10% glycerol, 1 mM DTT, 2 μ g/ml leupeptin, 5 μ g/ml aprotinin, 1 mM PMSF, 5 mM NaF, 10 mM *p*-nitrophenyl phosphate, and 1 mM Na₃VO₄). Cell lysates containing 20–30 μ g protein were subjected to reducing SDS–PAGE (12.5%). After electrophoresis, the gel was electroblotted in Tris–glycine buffer containing 40% methanol onto a reinforced nitrocellulose membrane (Schleicher–Schuell, Keene, NJ). The membrane was blocked with TBS-T buffer (10 mM Tris, pH 8.0, 150 mM NaCl, and 0.05% Tween 20) containing 5% milk powder for 1 h at room temperature and incubated with rabbit anti-mouse IgG against CREB (1:1000), or with mouse IgG anti-phosphorylated-CREB (1:500), in TBS-T containing 1% milk powder for 2 h at room temperature. The membrane was washed with TBS-T and incubated with secondary antibody: peroxidase-conjugated (goat anti-rabbit IgG or rat anti-mouse IgG) at 1:5000 dilution for 1 h at room temperature. After washing three times in TBS-T for 5 min each, and once in TBS for 5 min, the membrane was drained briefly and developed with the enhanced chemiluminescence system (ECL, Amersham).

Interaction of CBP with p65, cFos, and/or CREB was assessed as described previously [10,29] with some modifications. Endogenous p65 or CREB was immunoprecipitated from whole protein extract (200 mg/sample) by incubation with 1–2 μ g anti-p65, anti-cFos, or anti-CREB antibodies, for 4 h at 4 °C. The immune complexes were collected by incubation with 25 μ l protein A/G–Sepharose beads (Sigma) for 45 min at 4 °C. The beads were extensively washed with lysis buffer, twice with LiCl buffer (500 mM LiCl, 100 mM Tris–HCl, pH 7.6, and 0.1% Triton X-100), boiled in 1 × SDS sample buffer and separated by SDS–PAGE before blotting to a nitrocellulose membrane. The membrane was immunoblotted as described above using various dilutions of anti-CBP antibody. After detection with an appropriate secondary antibody conjugated peroxidase, proteins were visualized by enhanced chemiluminescence as described above.

Results and discussion

Under normal conditions, brain microglia, the ontogenetic and functional equivalents of mononuclear phagocytes in somatic tissues [1], are involved in immune surveillance and host defense against infectious agents. However, in response to brain injury, infection, or inflammation, microglia readily become activated, in a way similar to peripheral tissue macrophages, a process which includes differentiation and probably invasion and proliferation. Activation of microglia is a histopathological hallmark of several neurodegenerative diseases, including Alzheimer and Parkinson's diseases, multiple sclerosis, and the AIDS dementia complex [1].

Pathological microglial activation, in response to pro-inflammatory cytokines or antigens such as LPS, is believed to contribute to progressive damage in neurodegenerative diseases through the release of pro-inflammatory and/or cytotoxic factors. Hence, it is important to unravel mechanisms regulating microglia activation of inflamed brain parenchyma to provide insights into efficient therapeutic intervention. Secretion of pro-inflammatory products is followed later by the production of anti-inflammatory cytokines such as IL-10 and TGF β . Since the intensity and duration of an inflammatory process depend on the local balance between pro- and anti-inflammatory factors, several microglia-deactivating mechanisms are in place in normal circumstances. One such mechanism involves neural immune interactions, and particularly, the neuropeptides VIP and PACAP. Several recent reports indicated that VIP and PACAP inhibit the production of microglia-derived pro-inflammatory agents such as TNF α , IL-6, IL-1 β , NO, and chemokines, and stimulate the production of the anti-inflammatory cytokine IL-10 [24,25]. The regulation of these factors occurs at the transcriptional level and VIP/PACAP inhibition of NF- κ B-dependent gene activation plays a crucial role in such effect. The neuropeptides inhibit NF- κ B nuclear translocation and DNA binding, by inhibiting the I κ B kinase (IKK)-mediated I κ B phosphorylation/degradation in activated microglia [24,25].

In addition to DNA binding, the interaction of p65 with the coactivator CBP is essential for optimal NF- κ B transcriptional activity [9,10]. In addition to p65, CBP interacts with other factors, such as CREB, c-Jun, c-Fos, p53, glucocorticoid receptor, and the retinoid X receptor [6,7,13–16]. Changes in p65 phosphorylation or competition with other factors for the limiting quantities of nuclear CBP lead to changes in p65/CBP interactions. To address whether VIP and PACAP could be affecting the formation of p65/CBP complexes, microglia cells were stimulated with LPS or with TNF α in the absence or presence of VIP or PACAP, and total cell lysates were immunoprecipitated with antibodies against p65 or CREB and probed for the presence of CBP. LPS and TNF α stimulations result in the appearance of p65/CBP complexes (Fig. 1). 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. 1). Total CBP levels were not affected by either treatment. In addition, VIP and PACAP also reduce cFos/CBP complex formation (Fig. 1). These results show that VIP and PACAP increase CBP-binding to CREB, replacing p65/CBP with CREB/CBP complexes in activated microglia.

Phosphorylation of p65 strengthens the interaction with CBP [9,12]. However, VIP and PACAP failed to inhibit LPS-induced p65 phosphorylation (data not shown), arguing against the possibility that the neuro-

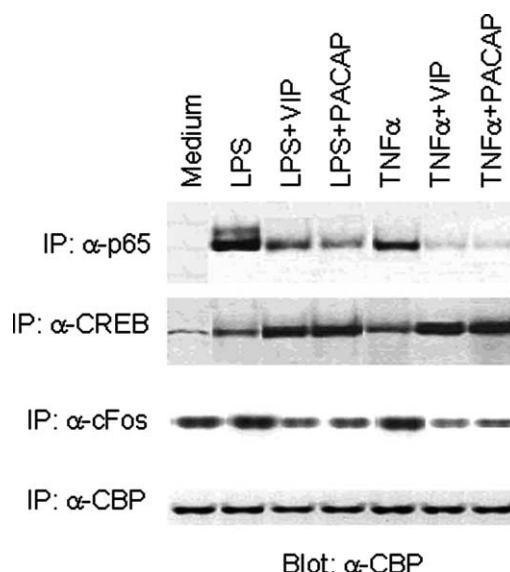


Fig. 1. VIP and PACAP promote CREB/CBP versus p65/CBP interactions in activated microglia. Microglia cells (5×10^6 cells) were incubated with medium (unstimulated), or activated with LPS (1 μ g/ml) or with TNF α (20 ng/ml), in the presence or absence of VIP (10^{-8} M) or PACAP (10^{-8} M) for 1 h. Whole cell extracts were subjected to immunoprecipitation with anti-CREB, anti-p65, or anti-cFos antibodies (IP) and analyzed by Western blot using an anti-CBP antibody as described in Materials and methods. Cell extracts immunoprecipitated and immunoblotted with anti-CBP antibodies were used as control for loading. One representative experiment of four independent assays is shown.

peptides inhibit p65/CBP interaction through this mechanism. However, CBP also binds to phosphorylated CREB and formation of CREB/CBP complexes will reduce the CBP available for complexing with p65 [9,11,13]. Since VIP/PACAP 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 and TNF α increase CREB phosphorylation slightly as compared to unstimulated controls (Fig. 2, upper panel). In contrast, VIP and PACAP strongly augment the levels of phosphorylated CREB (Fig. 2, upper panel). Total CREB levels were not affected by either treatment (Fig. 2, lower panel). Therefore, VIP and PACAP stimulate CREB phosphorylation/activation and the subsequent recruitment of CBP. A similar effect of VIP and PACAP on CREB phosphorylation and subsequent CREB-CBP versus p65-CBP interaction was previously obtained using activated human monocytes [30], where a functional correlation between the regulation of CBP interaction with p65 and CREB and the transcriptional activities of promoters controlled by these transcription factors was found. Unfortunately, transfection rates in microglia cells are very low and it is very difficult to determine transcriptional activity using reporter gene systems. However, transfection of the murine microglia

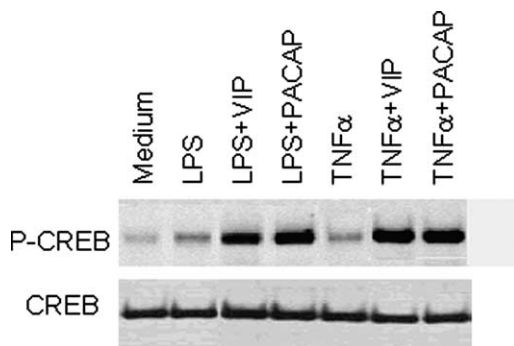


Fig. 2. VIP and PACAP stimulate CREB phosphorylation in activated microglia. Microglia cells (5×10^6 cells) were incubated with medium (unstimulated), or activated with LPS ($1 \mu\text{g/ml}$) or with $\text{TNF}\alpha$ (20 ng/ml), in the presence or absence of VIP (10^{-8} M) or PACAP (10^{-8} M) for 1 h. Whole cell extracts were analyzed by Western blot using anti-phosphorylated CREB or anti-CREB (used as control for loading). Data are representative of four different experiments.

cell line N6 with the $(\kappa\text{B})_4$ -reporter system [30] shows that VIP partially inhibited the LPS-induced NF- κB activation, and that cotransfection with increasing concentrations of p65 and CBP completely reversed the VIP/PACAP effect (not shown), suggesting a physiological role of the regulation of CBP interaction with p65 and CREB by VIP/PACAP in the NF- κB -dependent gene activation in stimulated microglia.

Three types of VIP/PACAP receptors have been cloned, i.e., VPAC1 and VPAC2 which express similar affinities for VIP and PACAP, and PAC1, the PACAP-preferring receptor which has an affinity approximately a thousand fold higher for PACAP [31]. Microglia have been shown to express VIP/PACAP-binding sites [32] and previous data demonstrate that, similar to rat microglia [32], mouse primary microglia and the microglia cell lines EOC and BV2 express VPAC1 and PAC1, but not VPAC2 [24]. Our previous studies identified VPAC1 as the major mediator of VIP/PACAP effects on microglia-derived cytokines [24,25]. I examined the role of VPAC1 in the modulation of the interaction of CBP with p65 or CREB and the stimulation of CREB phosphorylation by using a specific VPAC1-agonist and VPAC1-antagonist [33,34]. Also, since VPAC1 activates the adenylate cyclase, I determined the effect of the specific PKA inhibitor, H89. The VPAC1-antagonist and the PKA inhibitor completely reversed the effect of VIP on CREB phosphorylation, and the VPAC1-agonist entirely mimicked the effect of VIP (Fig. 3). In contrast, a PAC1-antagonist [35] failed to inhibit VIP effects (Fig. 3). A similar conclusion was reached for the effect of VIP on the preferential induction of CREB/CBP versus p65/CBP complexes (Fig. 3), suggesting that the VIP effects are mediated through VPAC1 and the cAMP/PKA pathway. This is supported by the fact that forskolin (a cAMP-inducing agent) exerts a similar effect with VIP (Fig. 3). However, the fact that although

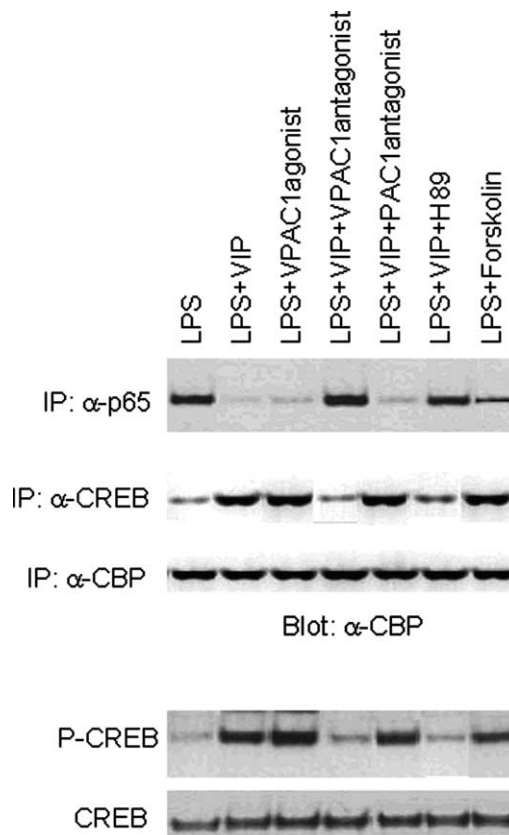


Fig. 3. Involvement of VPAC1 and cAMP/PKA in the VIP/PACAP modulation of p65/CBP and CREB/CBP complex formation and CREB phosphorylation. Microglia cells (5×10^6 cells) were stimulated with LPS ($1 \mu\text{g/ml}$) in the absence or presence of VIP (10^{-8} M), a VPAC1-agonist (10^{-8} M), or forskolin (10^{-6} M). The VPAC1-antagonist (10^{-6} M) or H89 (100 nM) was added simultaneously with VIP (10^{-8} M). After 1 h culture, whole cell extracts were analyzed for p65/CBP and CREB/CBP interactions and CREB phosphorylation as described in Figs. 1 and 2, respectively. One representative experiment of four is shown.

forskolin induced CREB phosphorylation and CBP-CREB interaction as efficiently as VIP, it was less efficient in disrupting CBP-p65 interaction (Fig. 3) could suggest that the cAMP pathway is necessary, but not sufficient for the inhibition of p65-CBP binding.

In conclusion, the present study shows that binding of VIP or PACAP to VPAC1 receptors inhibits, mainly through a cAMP-dependent mechanism, the LPS/ $\text{TNF}\alpha$ -induced formation of p65/CBP complexes in microglia, and that this event is directly related to the neuropeptide inhibition of NF- κB transcriptional activity on several pro-inflammatory genes. This may represent a significant element in the regulation of the inflammatory response in the CNS by the endogenous neuropeptides.

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