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Vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide inhibit the MEKK1/MEK4/JNK signaling pathway in endotoxin-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 proinflammatory factors. In the present study the effects of VIP/PACAP on the MEKK1/MEK4/JNK transduction pathway and on the subsequent changes in Jun family members, a transduction pathway clearly involved in the activation of microglia cells were examined. VIP/PACAP inhibit MEKK1 activity and the subsequent phosphorylations of MEK4, JNK, and c-Jun, which result in a decrease in the AP-1 binding and a marked change in the composition of AP-1 complexes from c-Jun/c-Fos to JunB/c-Fos. Furthermore, VIP stimulates JunB production in LPS-stimulated microglia. Both inhibition of the MEKK1/MEK4/JNK pathway, leading to a reduction in phosphorylated c-Jun, and the stimulation of JunB are mediated through the specific VPAC1 receptor and cAMP/PKA pathway. The VIP/PACAP interference with the stress-induced SAPK/JNK pathway in activated microglia may represent a significant element in the regulation of inflammatory response in the CNS by endogenous neuropeptides. © 2002 Elsevier Science (USA). All rights reserved.

Keywords: Neuroimmunomodulation; SAPK/JNK; Microglia; Neuropeptides; Inflammation

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 become readily 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's and Parkinson's diseases, multiple sclerosis, and the AIDS dementia complex [1]. Pathological microglial activation, in response to proinflammatory cytokines or antigens such as LPS, is believed to contribute to progressive damage in neurodegenerative diseases through the release of proinflammatory and/or cytotoxic factors, including TNFα, IL-1β, IL-6, IL-12, and nitric oxide (NO). Hence, it is important to unravel mechanisms regulating the microglia activation of inflamed brain parenchyma to provide insights into efficient therapeutic intervention. Secretion of proinflammatory products is later followed by the production of anti-inflammatory cytokines such as IL-10 and TGFβ. Since the intensity and duration of an inflammatory process depend on the local balance between pro- and anti-inflammatory factors, a number of regulatory molecules termed microglia-deactivating factors have been the focus of considerable research.

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 action on natural and acquired immunities [2], 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,

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respiratory burst, and chemotaxis [2], as well as LPS-induced IL-6, TNFα, IL-12, NO, and chemokine production [3–7]. Similarly, we have recently demonstrated that both neuropeptides act as two potent microglia-deactivating factors by inhibiting the production of LPS-induced proinflammatory mediators (Delgado et al., submitted). The inhibition of proinflammatory mediators is responsible, at least partially, for the protective effect of VIP and PACAP in vivo in a murine model for septic shock [8], and might contribute to their role as survival factors for neuronal cells in injury models ([9]; Delgado et al., submitted). The effects of VIP and PACAP on the expression of microglia-derived factors are exerted at a transcriptional level. However, although several of the transcriptional factors regulated by VIP and PACAP have been identified, the affected upstream transduction pathways are less clear. We have recently shown that VIP/PACAP inhibit IRF-1 transcription by interfering with the Jak1/STAT1 transduction pathway and inhibit NFκB translocation by preventing IkB phosphorylation and subsequent degradation (Delgado et al., submitted).

Stimulation of microglia leads to the activation of all three MAPK cascades, i.e., the mitogen-activated protein kinase (MAPK; ERK), the stress-activated protein kinase (JNK), and the p38 kinase [10,11]. JNK regulates the activity of several transcription factors such as c-Jun, ATF2, and ELK-1. JNK itself is activated in response to stress signals such as osmotic and heat shock, UV light, protein synthesis inhibitors, DNA-damaging drugs and proinflammatory cytokines [12,13]. The activation of JNK is mediated through phosphorylations of threonine and tyrosine residues by the upstream MAPK kinases MKK4/7 (MEK4/7) (reviewed in [14]). MKK4 activation is regulated in turn by phosphorylation of the upstream MAPK kinase kinase (MEKK1). Activation of the MEKK1/MKK4/JNK pathway by LPS is involved in the upregulation of several proinflammatory factors [14].

In this study, I examine the effects of VIP/PACAP on the MEKK1/MKK4/JNK cascade in activated microglia. I conclude that VIP and PACAP inhibit MEKK1, MKK4, and JNK activities, and the subsequent binding of the transcriptional factor AP-1, by changing the composition of the AP-1 complex from c-Jun/c-Fos to JunB/c-Fos. 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-His^{1}, D-Phe^{2}, K^{15}, R^{16}, L^{27}] VIP [3–7]-GRF [8–27] was donated by Dr. Patrick Robberecht (Universite Libre de Bruxelles, Belgium). Calphostin C, lipopolysaccharide (LPS from *Escherichia coli* 055:B5), and forskolin were purchased from Sigma Chemicals (St. Louis, MO) and H89 from ICN Pharmaceuticals (Costa Mesa, CA). Recombinant c-Jun kinase (JNK, 1–338)-tagged fusion protein and antibodies against JNK, c-Jun, JunB, c-Fos, and MEK4 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). c-Jun (1–89)-tagged fusion protein and Abs against phosphorylated MEK4 were purchased from New England Bio Labs (Beverly, MA).

Cell cultures. Microglial cell cultures were prepared as previously described [15]. Purified microglial cell cultures comprised 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). Microglial monolayers were stimulated with LPS (1 μg/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% CO_2.

Electrophoretic mobility shift assay (*EMSA*). Nuclear extracts were prepared by the mini-extraction procedure as previously described [4]. Oligonucleotides corresponding to the consensus AP-1 (5′-AGCTTGATGACTCAGC CGGAA-3′) were annealed after incubation for 5 min at 85 °C in 10 mM Tris–HCl, pH 8.0, 5 mM NaCl, 10 mM $MgCl_2$, and 1 mM DTT. Double-stranded oligonucleotides (50 ng) were end-labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase. I used 20,000–50,000 cpm of double-stranded oligonucleotides, corresponding to approximately 0.5 ng, for each reaction. The binding reaction mixtures (15 μl) contained: 0.5–1 ng DNA probe, 5 μg nuclear extract, 2 μg poly(dI–dC)·poly(dI–dC) and binding buffer (50 mM NaCl, 0.2 mM EDTA, 0.5 mM DTT, 5% glycerol, and 10 mM Tris–HCl, pH 7.5). The mixtures were incubated on ice for 15 min before adding the probe, followed by another 20 min at room temperature. Samples were loaded onto 4% nondenaturing polyacrylamide gels and electrophoresed in TGE buffer (50 mM Tris–HCl, pH 7.5, 0.38 M glycine, and 2 mM EDTA) at 100 V, followed by transfer to Whatman paper, drying under vacuum at 80 °C, and autoradiography. In competition with antibody supershift experiments, the nuclear extracts were incubated for 15 min at room temperature with the specific antibody (1 μg) or competing cold oligonucleotide (50-fold excess) before the addition of labeled probe.

Immunoblotting, *immunoprecipitation*, *and kinase assays*. Cell lysates were prepared by lysing 5×10^6 cells in lysis buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 2 mM EGTA, 50 mM glycerol phosphate, 1% Triton X-100, 10% glycerol, 1 mM DTT, 2 μg/ml leupeptin, 5 μg/ml aprotinin, 1 mM PMSF, 5 mM NaF, 10 mM *p*-nitrophenyl phosphate, and 1 mM Na_3VO_4). Cell lysates containing 20–30 μg 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 primary antibodies (rabbit IgG) against MEK4 (1:1000), JunB (1:500), or c-Jun (1:500), or with mouse IgG anti-phosphorylated MEK4 (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 chemiluminiscence system (ECL, Amersham).

Endogenous JNK and MEK4 were immunoprecipitated from cell lysates (150–250 μg/sample) by incubation with 0.5 μg of anti-JNK and anti-MEK4 Abs, respectively, for 2 h at 4 °C. The immune complexes were collected by incubation with protein A/G–Sepharose beads for 45 min at 4 °C. The beads were extensively washed with the lysis buffer, twice with LiCl buffer (500 mM LiCl, 100 mM Tris–HCl, pH 7.6, and 0.1% Triton X-100), and twice with kinase buffer (20 mM MOPS, pH 7.6, 2 mM EGTA, 10 mM $MgCl_2$, 1 mM DTT, 0.1% Triton X-100,

1 mM *p*-nitrophenyl phosphate, and 1 mM Na_3VO_4). The pelleted beads were resuspended in kinase buffer with 15 μM of ATP, 10 μCi [γ-^{32}P]ATP (3000 Ci/mmol, Amersham, Arlington, IL), and with 5 μg recombinant c-Jun (for JNK assay), or 10 μg recombinant JNK1 (for MEK4 assay). The kinase reaction was performed at 30 °C for 30 min and stopped with 15 μl 2XSDS sample buffer, boiled for 5 min, and subjected to SDS–PAGE (9%). Proteins were transferred onto a nitrocellulose membrane (Schleicher–Schuell) followed by autoradiography. The JNK and MEK4 activities were determined by incorporation of ^{32}P into their respective substrates (recombinant c-Jun- and JNK1-tagged fusion protein). The phosphorylated proteins were quantitated on a PhosphorImager. Equal loading was verified by immunoblotting of cell lysates as described above.

Results and discussion

VIP/PACAP inhibit the MEKK1/MEK4/JNK cascade

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 proinflammatory mediators [1]. Therefore, 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 proinflammatory agents such as TNFα, IL-6, IL-1β, NO, and chemokines, and stimulate the production of anti-inflammatory cytokine IL-10 [3–7]. The regulation of these factors occurs at the transcriptional level and several transcriptional factors including NFκB and IRF-1 were shown to be affected by VIP/PACAP (Delgado et al., submitted).

In this study I examined the effects of VIP/PACAP on the MEKK1/MEK4/JNK transduction pathway, responsible for the ultimate phosphorylation and activation of c-Jun and the subsequent transcription of several proinflammatory mediators [14]. LPS induces all three MAPK pathways in macrophages and microglia, i.e., the classical ras-raf-1-MEK1/2-Erk1/2, the stress-induced rac-MEKK1-MEK4/7-JNK1/2 pathway and the MKK3/6-p38 kinase pathway [10,11]. Activation of the JNK in response to LPS involves a cascade in which the upstream activator MEKK1 phosphorylates and activates MEK4, which in turn phosphorylates and activates JNK [16–18]. Phosphorylation of c-Jun by JNK increases the transactivating properties of this protein [14,19]. I first examined the JNK activity in whole cell extracts by using an immune complex kinase assay. Whereas c-Jun was weakly phosphorylated in unstimulated microglia, the c-Jun phosphorylation increased within 30 min and peaked 60 min after LPS stimulation (Fig. 1A, upper panel). Previous dose–response experiments established that the optimal concentrations of VIP and PACAP for the inhibition of microglia-derived cytokines were 10^{-7}–10^{-8} M. Therefore I used 10^{-8} M VIP or PACAP in all our experiments. Treatment with VIP or PACAP significantly reduced the JNK activity at all time points. In contrast, I observed no significant differences in the c-Jun protein levels (Fig. 1A, lower panel). Since c-

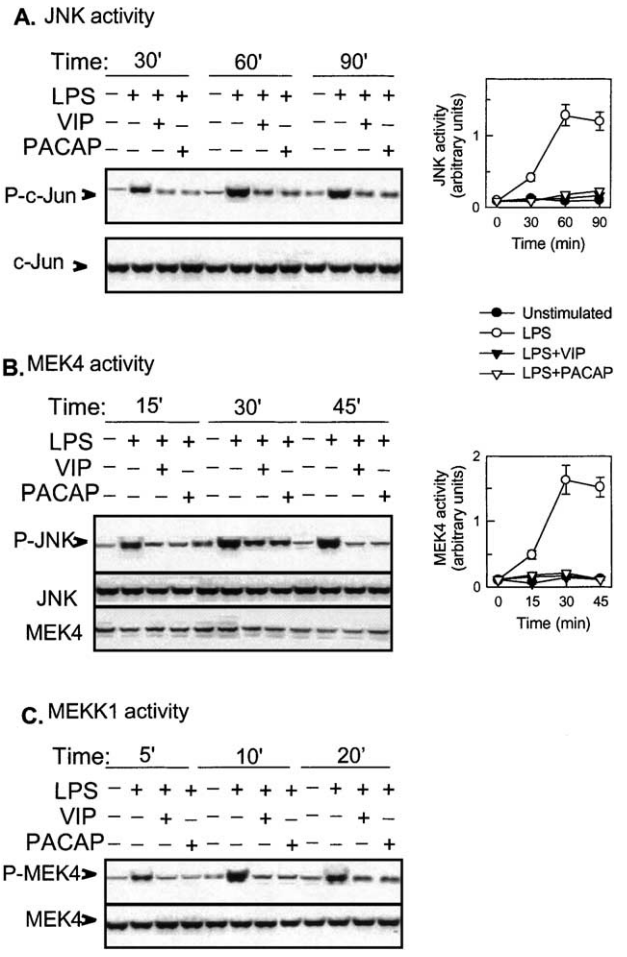

Fig. 1. VIP and PACAP inhibit the MEKK1/MEK4/JNK signaling pathway in LPS-stimulated microglia. Microglia cells (2×10^7 cells) were incubated with medium (unstimulated), or activated with LPS (1 g/ml), in the presence or absence of VIP (10^{-8} M) or PACAP (10^{-8} M). (A) VIP and PACAP inhibit JNK activity. At different time points JNK activity was assayed in an in vitro kinase assay with c-Jun as substrate (P-c-Jun). As control, the amounts of c-Jun were determined by immunoblotting with an anti-c-Jun Ab (c-Jun). One representative experiment of four independent assays is shown. *Right panel*, JNK activity is expressed as arbitrary densitometric units. Data are presented as means ± SD of four independent assays. (B) VIP and PACAP inhibit MEK4 activity. At 10, 20, and 45 min, MEK4 activity was assayed in an in vitro kinase assay with JNK as substrate (P-JNK). As control, the amounts of MEK4 and JNK were determined by immunoblotting. One representative experiment of four independent assays is shown. *Right panel*, MEK4 activity is expressed as arbitrary densitometric units. Data are presented as means ± SD of four independent assays. (C) VIP and PACAP inhibit the MEKK1 activity. At 5, 10, and 30 min, the MEKK1 activity was assayed by Western blot using a specific antibody against phosphorylated-MEK4. As control, the amounts of MEK4 were determined by immunoblotting. One representative experiment of four independent assays is shown.

Jun phosphorylation is the result of a cascade involving MEKK1/MEK4/JNK, I examined the effect of VIP and PACAP on the MEK4 activity in an in vitro kinase assay with JNK as substrate. Treatment with LPS resulted in a time-dependent increase in MEK4 activity (Fig. 1B). VIP and PACAP significantly inhibited the MEK4-mediated phosphorylation of JNK without affecting the MEK4 protein levels (Fig. 1B). Finally, I investigated the effect of VIP and PACAP on the MEKK1 activity, the upstream regulator in this kinase cascade. Since MEK4 is phosphorylated by MEKK1, I determined the levels of phosphorylated MEK4 in whole cell extracts by Western blot. LPS stimulated in a time-dependent manner the phosphorylation of MEK4 and VIP or PACAP significantly reduced the levels of phosphorylated MEK4 (Fig. 1C). I conclude that the VIP/PACAP inhibition of c-Jun phosphorylation is mediated through the effects of the two neuropeptides on the MEKK1/MEK4/JNK cascade.

Role of VPAC1 and cAMP in the inhibition of MEKK1/MEK4/JNK by VIP/PACAP

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 [20]. Our previous studies identified VPAC1 as the major mediator of VIP/PACAP effects on microglia-derived cytokines (Delgado et al., submitted). I examined the role of VPAC1 in the inhibition of the MEKK1/MEK4/JNK pathway by using a specific VPAC1 antagonist [21]. Also, since VPAC1 activates the adenylate cyclase, I determined the effect of the specific PKA inhibitor, H89. Both the VPAC1-antagonist and H89 reversed the inhibitory effects of VIP on the LPS-induced phosphorylation of MEK4, JNK, and c-Jun (Fig. 2), suggesting that the VIP inhibition is 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. 2).

Reduction in AP-1 binding by VIP and PACAP

As a consequence of JNK inhibition, the levels of phosphorylated c-Jun decrease in microglia treated with VIP or PACAP. Changes in c-Jun lead to changes in the composition of various transcriptional complexes. The most common are the Jun/Fos heterodimers that bind to the TPA-response elements (AP-1 sites) in the promoters of various genes [22]. In addition to c-Jun and c-Fos, other members of these families may form heterodimers with different levels of transactivating activity. For example, while heterodimers containing c-Jun are good transactivators, those containing JunB may act as

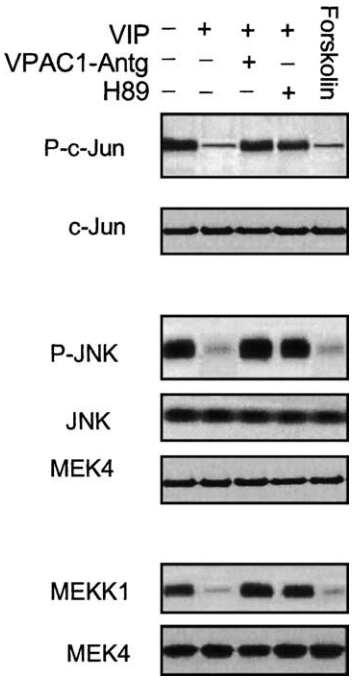

Fig. 2. Involvement of VPAC1 and cAMP/PKA in the VIP/PACAP inhibition of the MEKK1/MEK4/JNK pathway. Microglia cells (2×10^7 cells) were stimulated with LPS (1 μg/ml) in the absence (lane 1) or presence of VIP (10^{-8} M, lanes 2, 3, and 4) or forskolin (10^{-6} M, lane 5). The VPAC1-antagonist (10^{-6} M, lane 3) or H89 (100 nM, lane 4) was added simultaneously with VIP (10^{-8} M). The kinase activities of JNK, MEK4, and MEKK1 were assayed after 30, 20, and 15 min incubation, respectively, as described in Fig. 1. As control, the amounts of JNK and MEK4 were determined by immunoblotting. One representative experiment of four is shown.

transcriptional inhibitors. Since VIP and PACAP inhibit c-Jun phosphorylation, I examined whether the neuropeptides regulate the composition of AP-1 complexes, and their binding capacity. Treatment of microglia cells with LPS led to a strong AP-1 binding compared to unstimulated cells and treatment with VIP or PACAP decreased this binding (Fig. 3A, left panel). The oligonucleotide specificity was confirmed by the lack of binding in the presence of excess unlabeled AP-1 oligonucleotide (AP-1), in contrast to a nonspecific oligonucleotide (NFκB) (Fig. 3A, right panels). Supershift experiments with specific antibodies were used to determine the composition of the AP-1-binding complexes. In LPS-stimulated cells, anti-c-Jun or anti-c-Fos reduced significantly the intensity of the AP-1 band and led to the appearance of slower migrating bands. The anti-JunB Ab had a much lesser effect. No supershift was observed using an irrelevant Ab (anti-p65 Ab) (Fig. 3B). Treatment with VIP or PACAP results in the replacement of c-Jun with JunB, since no supershift was observed with anti-c-Jun Abs, whereas a major supershift occurred with anti-JunB Abs (Fig. 3B). In contrast, VIP and PACAP did not affect c-Fos binding to the AP-1 element (Fig. 3B). These results indicate that VIP/

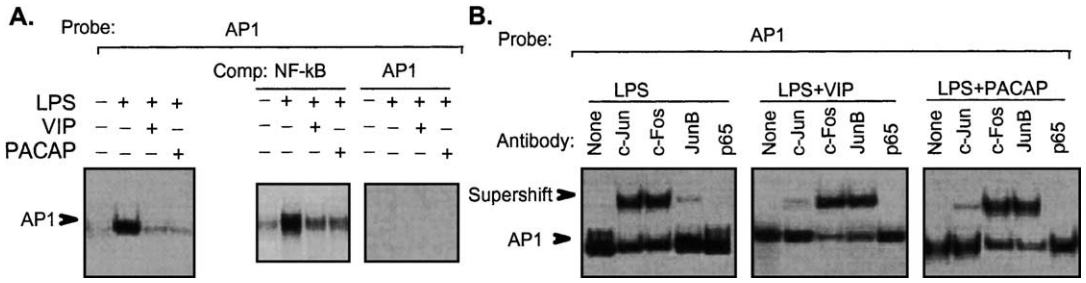

Fig. 3. VIP and PACAP alter the composition of AP-1-binding complexes. (A) VIP and PACAP decrease AP-1 binding. Microglia cells (2×10^7 cells) were incubated with medium (unstimulated) or activated with LPS (1 μg/ml), in the presence or absence of VIP (10^{-8} M), or PACAP (10^{-8} M). After 2 h incubation, nuclear extracts were isolated and AP-1 binding was assessed by EMSA using a radiolabeled oligonucleotide containing the consensus AP-1 site. Specificity was assessed by adding a 50-fold excess of unlabeled homologous (AP-1), or nonhomologous (NFκB) oligonucleotide (Comp). (B) Identification of the proteins bound to the AP-1 site. Nuclear extracts were incubated with polyclonal Abs (1 μg) against p65, c-Jun, c-Fos, or JunB for 20 min before adding the probe. Arrow indicates the AP-1 binding. Supershifted c-Fos-, JunB-, and c-Jun-specific bands are also indicated. Similar results were observed in four independent experiments.

PACAP induce a change in the AP-1 composition from c-Jun- to Jun B-containing complexes and a decrease in the AP-1 binding.

VIP and PACAP increase JunB protein levels in LPS-stimulated macrophages

To determine whether VIP and PACAP increase JunB expression in LPS-stimulated microglia, I measured the JunB protein levels by Western blot. Although no JunB protein was detectable in unstimulated cells, increased levels of JunB are present in LPS-stimulated cells. VIP and PACAP strongly stimulate the expression of JunB protein (Fig. 4A). The stimulatory effect of VIP was reversed by the VPAC1 antagonist and H89 (Fig. 4B), again suggesting the participation of VPAC1 and of the cAMP/PKA pathway. This is supported by the fact that forskolin has a similar stimulatory effect on JunB (Fig. 4B). JunB was classified as a negative transcriptional regulator [23], based on amino acid changes in its DNA-binding and dimerization domains, which lead to JunB/c-Jun heterodimers with low DNA-binding activity [24]. Alternatively, it has been proposed that JunB competes with c-Jun for JNK binding, leading to a reduction in c-Jun activation. This is supported by the fact that JunB contains an intact JNK-binding domain and can be phosphorylated by JNK. In either case, the VIP/PACAP upregulation of JunB leading to the replacement of the transcriptionally active c-Jun in the AP-1 complexes could result in the silencing of certain promoters. A similar observation was reported for LPS-stimulated macrophages and activated T cells [25,26].

In conclusion, the present study shows that binding of VIP or PACAP to VPAC1 receptors inhibits the LPS-stimulated JNK/AP-1 transactivating signaling in microglia by inhibiting the MEKK1 activity through a cAMP-dependent mechanism. In addition, VIP increases the levels of JunB, leading to the replacement of c-Jun with JunB in complexes binding to the AP-1 sites, and to a decrease in binding and subsequent transactivating activity. The MEKK1/MEK1/JNK pathway may not be the only MAP kinase pathway affected by VIP and PACAP. Since one of the major effects of VIP/PACAP is the inhibition of NFκB translocation and since LPS activation of NFκB in microglia cells appears to be mediated through the p38 MAPK, it is tempting to speculate that VIP/PACAP might also affect the p38 MAPK pathway.

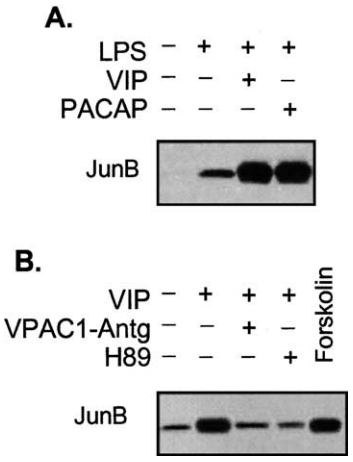

Fig. 4. VIP and PACAP stimulate the JunB protein expression in LPS-stimulated microglia. (A) Microglia cells (2×10^7 cells) were incubated with medium (unstimulated) or activated with LPS (1 μg/ml), in the presence or absence of VIP (10^{-8} M) or PACAP (10^{-8} M). After 2 h incubation, the JunB protein was analyzed by Western blot. One representative experiment of four is shown. (B) VIP-induced JunB expression is mediated through VPAC1 and cAMP/PKA. Microglia cells (2×10^7 cells) was stimulated with LPS (1 μg/ml) in the absence (lane 1) or presence of VIP (10^{-8} M, lane 2) or forskolin (10^{-6} M, lane 5). VPAC1-antagonist (10^{-6} M, lane 3) or H89 (100 nM, lane 4) were added simultaneously with VIP (10^{-8} M). The expression of JunB protein was assayed 90 min later by Western blot. One representative experiment of three is shown.

Acknowledgment

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References

[1] D.W. Dixon, L.A. Mattiace, K. Kure, K. Hutchins, X. Lyman, C.F. Brosnan, Microglia in human disease, with an emphasis on acquired immune deficiency syndrome, Lab. Invest. 64 (1991) 135–156.

[2] R.P. Gomariz, C. Martinez, C. Abad, J. Leceta, M. Delgado, Immunology of VIP: a review and therapeutical perspectives, Curr. Pharm. Des. 7 (2001) 89–111.

[3] M. Delgado, D. Pozo, C. Martinez, J. Leceta, J.R. Calvo, D. Ganea, R.P. Gomariz, Vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide inhibit endotoxin-induced TNFα production by macrophages: in vitro and in vivo studies, J. Immunol. 162 (1999) 2358–2367.

[4] M. Delgado, E.J. Munoz-Elias, Y. Kan, I. Gozes, M. Fridkin, D.E. Brenneman, R.P. Gomariz, D. Ganea, Vasoactive intestinal peptide and pituitary adenylate cyclase activating polypeptide inhibit TNFα transcriptional activation by regulating NF-κB and CREB/c-Jun, J. Biol. Chem. 273 (1998) 31427–31436.

[5] M. Delgado, E.J. Munoz-Elias, R.P. Gomariz, D. Ganea, VIP and PACAP prevent inducible nitric oxide synthase transcription in macrophages by inhibiting NF-κB and interferon regulatory factor 1 activation, J. Immunol. 162 (1999) 4685–4696.

[6] M. Delgado, E.J. Munoz-Elias, R.P. Gomariz, D. Ganea, VIP and PACAP inhibit IL-12 production in LPS-stimulated macrophages. Subsequent effect on IFNγ synthesis by T Cells, J. Neuroimmunol. 96 (1999) 167–181.

[7] M. Delgado, D. Ganea, Inhibition of endotoxin-induced macrophage chemokine production by VIP and PACAP in vitro and in vivo, J. Immunol. 167 (2001) 966–975.

[8] M. Delgado, C. Martinez, D. Pozo, J.R. Calvo, J. Leceta, D. Ganea, R.P. Gomariz, VIP and PACAP protect mice from lethal endotoxemia through the inhibition of TNFα and IL-6, J. Immunol. 162 (1999) 1200–1205.

[9] S.I. Said, Molecules that protect: the defense of neurons and other cells, J. Clin. Invest. 97 (1996) 99–103.

[10] H. Pyo, I. Jou, S. Jung, S. Hong, E.H. Joe, Mitogen-activated protein kinases activated by lipopolysaccharide and beta-amyloid in cultured rat microglia, Neuroreport 9 (1998) 871–874.

[11] P. Zhang, E.L. Hogan, N.R. Bhat, Activation of JNK/SAPK in primary glial cultures: II. Differential activation of kinase isoforms corresponds to their differential expression, Neurochem. Res. 23 (1998) 219–225.

[12] B. Derijard, M. Hibi, I.J. Wu, T. Barrett, B. Su, T. Deng, M. Karin, R.J. Davis, JNK1: a protein kinase stimulated by UV light and Ha–Ras that binds and phosphorylates the c-Jun activation domain, Cell 76 (1994) 1025–1037.

[13] Z. Galcheva-Gargova, B. Derijard, I.H. Wu, R.J. Davis, An osmosensing signal transduction pathway in mammalian cells, Science 265 (1994) 806–808.

[14] M.H. Cobb, MAP kinase pathways, Prog. Biophys. Mol. Biol. 71 (1999) 479–500.

[15] C.C. Chao, T.W. Molitor, H. Shuxian, Neuroprotective role of IL-4 against activated microglia, J. Immunol. 151 (1993) 1473–1481.

[16] I. Sanchez, R.T. Hughes, B.J. Mayer, K. Yee, J.R. Woodgett, J. Avruch, J.M. Kyriakis, L.I. Zon, Role of SAPK/ERK kinase 1 in the stress-activated pathway regulating transcription factor c-Jun, Nature 372 (1994) 794–798.

[17] J.S. Sanghera, S.L. Weinstein, M. Aluwalia, J. Girn, S.L. Pelech, Activation of multiple proline-directed kinases by bacterial lipopolysaccharide in murine macrophages, J. Immunol. 156 (1996) 4457–4465.

[18] A. Lin, A. Minden, H. Martinetto, F.X. Claret, C. Langer-Carter, F. Mercurio, G.L. Johnson, M. Karin, Identification of a dual specificity kinase that activates the Jun kinases and p38-Mpk2, Science 268 (1995) 286–290.

[19] B. Pulverer, J.M. Kryiakis, J. Avruch, E. Nikolakaki, J.R. Woodgett, Phosphorylation of c-jun mediated by MAP kinases, Nature 353 (1991) 6760–6764.

[20] A.J. Harmar, A. Arimura, I. Gozes, L. Journot, M. Laburthe, J.R. Pisegna, S.R. Rawlings, P. Robberecht, S.I. Said, S.P. Sreedharan, S.A. Wank, J.A. Washeck, Nomenclature of receptors for vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase activating polypeptide (PACAP), Pharmacol. Rev. 50 (1998) 625–627.

[21] P. Gourlet, P. De Neef, J. Cnudde, M. Waelbroeck, P. Robberecht, In vitro properties of a high affinity selective antagonist of the VIP1 receptor, Peptides 18 (1997) 1555–1560.

[22] M. Karin, Z.-G. Liu, E. Zandi, AP-1 function and regulation, Curr. Opin. Cell. Biol. 9 (1997) 240–246.

[23] R. Chiu, P. Angel, M. Karin, Jun-B differs in its biological properties from, and is a negative regulator of, c-Jun, Cell 59 (1989) 979–986.

[24] T. Deng, M. Karin, JunB differs from c-Jun in its DNA-binding and dimerization domains, and represses c-Jun by formation of inactive heterodimers, Genes Dev. 7 (1993) 479–490.

[25] H.-Y. Wang, X-M. Jiang, D. Ganea, Vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide inhibit interleukin 2 transcription by decreasing c-Jun and increasing JunB expression in T cells, J. Neuroimmunol. 104 (2000) 68–78.

[26] M. Delgado, D. Ganea, Vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide inhibit the MEKK1/MEK4/JNK signaling pathway in LPS-stimulated macrophages, J. Neuroimmunol. 110 (2000) 97–105.