Vasoactive Intestinal Peptide and Pituitary Adenylyl Cyclase-Activating Polypeptide Inhibit Tumor Necrosis Factor-α Production in Injured Spinal Cord and in Activated Microglia via a cAMP-Dependent Pathway

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Tumor necrosis factor-α (TNF-α) production accompanies CNS insults of all kinds. Because the neuropeptide vasoactive intestinal peptide (VIP) and the structurally related peptide pituitary adenylyl cyclase-activating polypeptide (PACAP) have potent anti-inflammatory effects in the periphery, we investigated whether these effects extend to the CNS. TNF-α mRNA was induced within 2 hr after rat spinal cord transection, and its upregulation was suppressed by a synthetic VIP receptor agonist. Cultured rat microglia were used to examine the mechanisms underlying this inhibition because microglia are the likely source of TNF-α in injured CNS. In culture, increases in TNF-α mRNA resulting from lipopolysaccharide (LPS) stimulation were reduced significantly by 10^{-7} M VIP and completely eliminated by PACAP at the same concentration. TNF-α protein levels were reduced 90% by VIP or PACAP at 10^{-7} M. An antagonist of $VPAC_1$ receptors blocked the action of VIP and PACAP, and a PAC_1 antagonist blocked the action of PACAP. A direct demonstration of VIP binding on microglia and the existence of mRNAs for $VPAC_1$ and PAC_1 (but not $VPAC_2$) receptors argue for a receptor-mediated effect. The action of VIP is cAMP-mediated because (1) activation of cAMP by forskolin mimics the action; (2) PKA inhibition by H89 reverses the neuropeptide-induced inhibition; and (3) the lipophilic neuropeptide mimic, stearyl-norleucine^{17} VIP (SNV), which does not use a cAMP-mediated pathway, fails to duplicate the inhibition. We conclude that VIP and PACAP inhibit the production of TNF-α from activated microglia by a cAMP-dependent pathway.

Key words: VIP; PACAP; TNF-α; microglia; cAMP; PKA; spinal cord injury; LPS

The proinflammatory cytokine tumor necrosis factor-α (TNF-α) is upregulated during the acute-phase response to CNS injury (Shohami et al., 1994; Yakovlev and Faden, 1994; C. Wang et al., 1996; Bartholdi and Schwab, 1997; Uno et al., 1997; Streit et al., 1998; Hart et al., 1999). Its release exacerbates acute CNS insult by promoting gliosis (Selmaj et al., 1991), inhibiting astrocytic glutamate uptake (Fine et al., 1996), and inducing apoptosis, particularly in oligodendrocytes (D'Souza et al., 1996; Hisahara et al., 1997), thereby contributing to damaging demyelination. Inhibition of TNF-α action in the early phases of traumatic CNS injury may yield a salutary clinical outcome.

Vasoactive intestinal peptide (VIP) and its related neuropeptide pituitary adenylyl cyclase-activating polypeptide (PACAP) have immunomodulatory actions on peripheral immune cells. VIP and PACAP inhibit proliferation and interleukin-2 (IL-2), IL-4, and IL-10 secretion of stimulated T-cells (Boudard and Bastide, 1991; Tang et al., 1995; Martinez et al., 1996; H. Wang et al., 1996) and downregulate natural killer cell activity (Sirianni et al., 1992). Working via both cAMP-dependent and -independent pathways, they inhibit several functions of activated peripheral macrophages, including phagocytosis and the production of TNF-α, IL-6, IL-12, and nitric oxide (Ichinose et al., 1994; Delgado et al., 1998, 1999a,b,d,e; Martinez et al., 1998), while enhancing the production of the anti-inflammatory IL-10 (Delgado et al., 1999c). Moreover, the rapid upregulation of VIP and PACAP in axotomized neurons in both the peripheral nervous system (PNS) and CNS suggests their involvement in the acute neuronal response to injury (Shehab and Atkinson, 1986; Hyatt-Sachs et al., 1993; Rao et al., 1993; Zhang et al., 1993, 1996; Klimaschewski et al., 1996; Moller et al., 1997; Zhou et al., 1999). In fact, VIP and PACAP have been shown to promote neuronal survival in culture (Brenneman et al., 1985; Klimaschewski et al., 1995; Campard et al., 1997). To determine whether these anti-inflammatory actions extend to the CNS, we have examined the effects of VIP agonist activity on TNF-α production induced by a spinal cord transection and have found that it inhibits the acute expression of TNF-α that follows such a lesion.

Inflammatory cytokines are upregulated in lesioned CNS before the invasion of neutrophils and monocytes (Woodroofe et al., 1991; C. Wang et al., 1996, 1997; Bartholdi and Schwab, 1997; Hayashi et al., 1997; Streit et al., 1998), implicating a resident cellular source. Although neurons and astrocytes may express inflammatory cytokines under certain conditions (Breder et al., 1988, 1993; Sharif et al., 1993; Liu et al., 1994), activated microglia are primary producers of proinflammatory cytokines (Sawada et al., 1989; Lee et al., 1993; Sébire et al., 1993; Lafortune et al., 1996). Furthermore, TNF-α immunoreactivity and mRNA in injured CNS are localized almost exclusively in microglia (Bartholdi and Schwab, 1997; Kita et al., 1997; Uno et al., 1997;

Received Oct. 1, 1999; revised Feb. 29, 2000; accepted March 1, 2000.

This work is supported by grants from the Christopher Reeve Paralysis Foundation (R.P.H.), the Charles and Johanna Busch Foundation (G.M.J.), Johnson & Johnson (W-K.K.), and Public Health Service AI 41786-02 (D.G.).

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Bruccoleri et al., 1998). Because microglia are, therefore, the most likely cellular source of TNF-α, we have used primary cultures of enriched microglia to examine the possible mechanisms by which VIP inhibits TNF-α expression.

MATERIALS AND METHODS

Reagents. VIP and PACAP-38 were from Novabiochem (Laufelfingen, Switzerland). The $VPAC_1$ receptor agonist (Lys^{15}, Arg^{16}, Leu^{27})VIP(1–7)-GRF(8–27) and the $VPAC_1$ receptor antagonist (Ac-His^1,D-Phe^2,Lys^{15},Arg^{16},Leu^{27})VIP(3–7)-GRF(8–27) were generously provided by Patrick Robberecht (Université Libre de Bruxelles, Belgium). The PAC_1 receptor antagonist $PACAP_{6-38}$ was purchased from Peninsula Laboratories (Belmont, CA). The lipophilic VIP analog stearyl-$norleucine^{17}$ VIP (SNV) was kindly provided by Illana Gozes (Tel Aviv University, Israel) and Mati Fridkin (Weizmann Institute of Science, Israel). Lipopolysaccharide (LPS) from *Escherichia coli* 055:B5 (Sigma, St. Louis, MO) was resuspended in sterile PBS and stored at −20°C. Forskolin (Sigma) and *N*-[2-(*p*-bromocinnamyl-amino)ethyl]-5-iso-quinolinesulfonamide (H89; ICN Pharmaceuticals, Costa Mesa, CA) were dissolved in DMSO (Sigma).

Spinal cord transection. To study effects of radical spinal cord injury, we freshly isolated upper cervical spinal cord from uninjured adult Sprague Dawley rats and cut it into 1 mm slices. The spinal cord slices were incubated for 2 hr at 37°C in serum-free N2 medium (Opti-MEM containing 1% N2 supplement; Life Technologies, Grand Island, NY) with and without the synthetic $VPAC_1$ receptor agonist. The slices were frozen for subsequent RNA extraction.

Cell cultures. Rat microglial-enriched cultures were prepared by using a slight modification of previously published methods (Jonakait et al., 1996; Wei and Jonakait, 1999). Cerebral cortices dissected from pups (postnatal day 1 or 2) were freed of meninges, minced manually, and triturated slowly with a pipette in N2 medium. The cells were plated into 25 cm^2 flasks at the concentration of one side of cortex/3.5 ml serum-containing medium (SCM) containing DMEM/F-12 (1:1), 25 U/ml of penicillin, 25 μg/ml of streptomycin, 0.6% D-glucose, and 10% fetal bovine serum. The mixed glial cultures were maintained by changing the SCM twice a week. After 2 weeks the flasks were agitated vigorously on a rotary shaker at 350 rpm for 10 min. The medium was collected in a sterile culture tube and centrifuged at 900 rpm for 5 min. Cells were seeded onto 48-well plates at a density of 1×10^5 cells/well per 400 μl for the determination of TNF-α protein or onto 6-well plates at a density of 1×10^6 cells/well per 2 ml for total RNA extraction. Cells were allowed to adhere to the substrates at 37°C for 3 hr. The plates were tapped gently to remove loosely adhering oligodendrocytes. The purity of the resulting enriched microglial culture was confirmed by DiI-Ac-LDL (Biomedical Technologies, Stoughton, MA) staining; >95% of cells stained positively.

The resulting monolayer of purified microglia was grown in SCM/N2 (1:1) for 24 hr. After 24 hr the medium was changed to N2 with or without additives. The application of N2 was designated t_0. When using the cAMP-dependent protein kinase (protein kinase A; PKA) inhibitor H89, we pretreated the cultures with the inhibitor for 30 min before t_0. The medium was removed, and N2 medium containing H89 and additives was added at t_0.

For some experiments a rat microglial cell line (HAPI; Cheepsunthorn et al., 1999) was used and treated exactly like primary microglial cells. It was obtained by arrangement with James R. Connor (Penn State University, College of Medicine, Hershey, PA).

TNF-α determinations. The supernatants were collected and stored at 4°C or, if not used immediately, frozen at −80°C until assayed for TNF-α secretion. TNF-α levels were determined by a TNF-α ELISA kit (BioSource, Camarillo, CA). The sensitivity of the assay was 15.6 pg/ml. The standards were performed in duplicate. For statistical analysis a single treatment was performed on three or four individual wells. Data are expressed as the mean pg ± SEM of TNF-α produced per milliliter for each treatment.

Reverse transcriptase-PCR (RT-PCR) and ribonuclease protection assay (RPA). Total RNA was extracted by using guanidinium thiocyanate-phenol (Chomczynski and Sacchi, 1987). For RT-PCR, 1 μg of total RNA was reverse-transcribed to complementary DNA (cDNA) by M-MLV reverse transcriptase (Life Technologies) at 42°C for 1 hr. The cDNA was amplified by Pyrostase polymerase (Molecular Genetic Resource, Tampa, FL). VIP receptor primers were those used previously by Delgado et al. (1998).

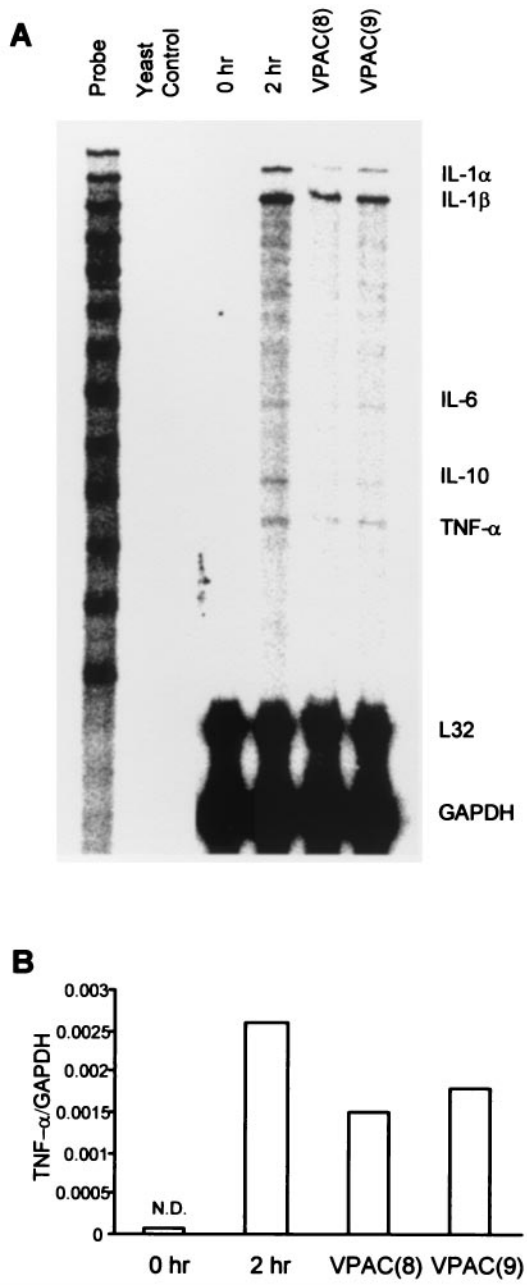

Figure 1. Spinal cord transection induces inflammatory cytokine mRNA expression. Freshly isolated spinal cord slices were incubated with or without 10^{-8} M or 10^{-9} M of a synthetic $VPAC_1$ agonist [denoted as *VPAC(8)* or *VPAC(9)*, respectively] for 2 hr. RNA was extracted and assayed by RPA for cytokine mRNAs (*A*). The lane marked *Probe* is a probe set untreated with RNase. The lanes on the *right* are protected fragments resulting from RNase treatment. In *B*, the levels of TNF-α mRNA were quantified by using a PhosphorImager with IPLab Gel H software and expressed as a ratio of TNF-α mRNA to GAPDH mRNA. These ratios were assigned arbitrary units for comparison. At time *0*, TNF-α mRNA is not detectable (*N.D.*). This experiment was repeated with virtually identical results.

Four micrograms of total RNA were used for RPA with a multiprobe RPA kit (RiboQuant; PharMingen, San Diego, CA). Protected fragments were resolved on a 5% denaturating polyacrylamide gel containing 8 M urea. The radiolabeled band of TNF-α was detected and quantitated with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) with densitometry software (IPLab Gel H). Data are expressed as a ratio of TNF-α mRNA normalized to the mRNA of the housekeeping gene glyceralde-

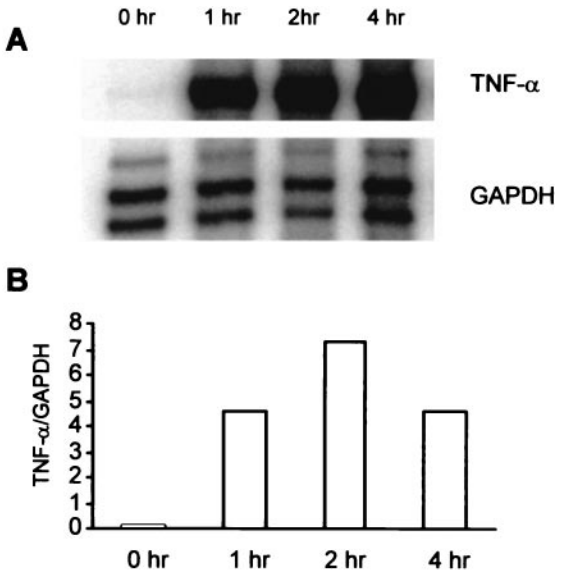

Figure 2. LPS induces TNF-α mRNA in microglia. *A*, Cultured microglia were treated with LPS (100 ng/ml) for various times, and RNA was assayed by RPA as described in Figure 1. *B*, TNF-α mRNA was expressed as a ratio to GAPDH mRNA and plotted as arbitrary units. TNF-α mRNA was elevated as early as 1 hr after LPS treatment.

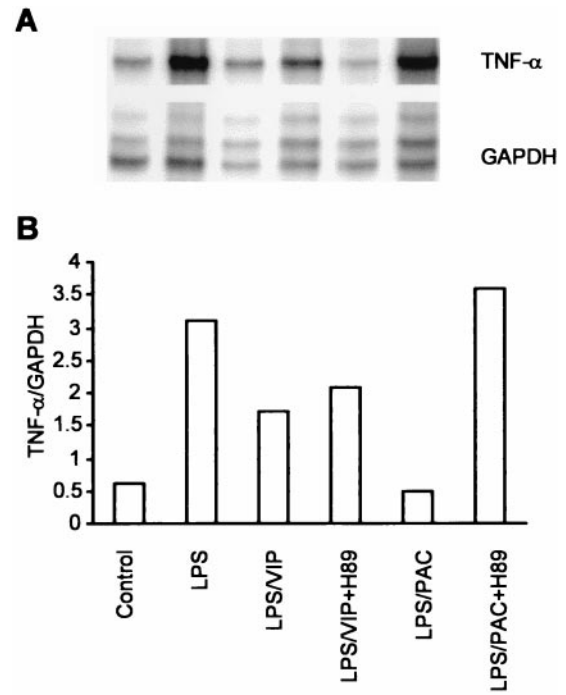

Figure 3. VIP and PACAP inhibit the LPS-induced increase in TNF-α mRNA in microglia. Cultured microglia were treated for 3 hr with LPS (100 ng/ml) with or without VIP or PACAP at 10^{-7} M or with the neuropeptides after a 30 min pretreatment with H89 (10^{-6} M). *A*, RPA analysis of TNF-α mRNA was performed as described in Figure 1. *B*,TNF-α mRNA was measured in arbitrary units and expressed as a ratio to GAPDH mRNA for comparisons among treatments.

hyde phosphate dehydrogenase (GAPDH). The lower molecular weight of the GAPDH-protected band usually results in RNase "nibbling," leading to the appearance of multiple bands. According to the manufacturer's recommendation, the most intense of three GAPDH bands was used for quantification.

Immunohistochemistry. The expression of VIP receptors on microglia was examined immunohistochemically. Primary microglial or HAPI cells were seeded onto glass coverslips at a density of 2×10^5 cells per coverslip. At t_0 the cells were incubated for 2 hr with LPS (100 ng/ml). Biotinylated VIP (10^{-10} M; Peninsula Laboratories) was added either simultaneously with LPS or after 1.5 hr. Cells were washed with ice-cold PBS and fixed in 4% paraformaldehyde, pH 7.4, for 10 min. For the simultaneous demonstration of VIP binding on OX-42-positive microglia, the cells were incubated overnight with mouse anti-rat monoclonal antibody against CD11b/c (5 μg/ml; clone OX-42; PharMingen). The secondary antibody was FITC-conjugated goat anti-mouse Ig (diluted at 1:500; PharMingen). Biotinylated VIP was visualized with Texas Red-conjugated avidin D (1:40; Vector Laboratories, Burlingame, CA). The signal was reinforced by exposure to a biotinylated anti-avidin D (3 μg/ml; Vector Laboratories) with a second exposure to Texas Red-conjugated avidin D. Images were collected by using the MRC-1024 laser-scanning confocal microscope system (Bio-Rad, Hercules, CA).

RESULTS

$VPAC_1$ receptor agonist suppresses TNF-α gene expression in a culture model of spinal cord injury

To examine the neuropeptide regulation of TNF-α gene expression that follows spinal cord injury, we incubated 1 mm slices of freshly isolated rat spinal cords in N2 medium for 2 hr with or without a $VPAC_1$ receptor agonist. Proinflammatory cytokines are expressed in spinal cord within 1–2 hr after traumatic injury *in vivo* (C. Wang et al., 1996, 1997; Bartholdi and Schwab, 1997; Hayashi et al., 1997; Streit et al., 1998; Hart et al., 1999), and this temporal profile of cytokine production is mimicked in spinal cord slices (Hart et al., 1999). Total RNA from the spinal cord slices was analyzed with a multiprobe RPA for cytokine mRNAs (see Materials and Methods). Consistent with the data of others (Bartholdi and Schwab, 1997; Streit et al., 1998; Hart et al., 1999), TNF-α mRNA was undetected in uninjured cords, but spinal cord transection produced an elevation of mRNAs for TNF-α as well as IL-1α, IL-1β, and IL-6 within 2 hr (Fig. 1). Inclusion of the synthetic $VPAC_1$ receptor agonist (10^{-8} M) inhibited TNF-α mRNA expression by as much as 50%. Inhibitory effects also were seen on IL-1α, IL-6, and IL-10.

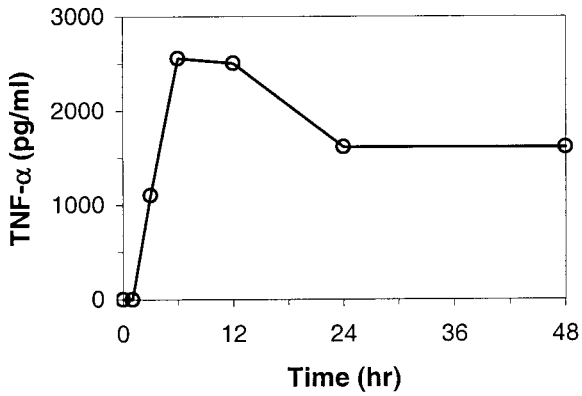

Figure 4. LPS induces TNF-α protein accumulation over time. Cultured microglia were stimulated with 100 ng/ml of LPS. The supernatants were collected at different times and assayed for TNF-α protein accumulation by ELISA. Each *point* represents the average of TNF determinations (pg/ml) in duplicate cultures. Cells cultured without LPS did not produce detectable amounts of TNF (<15.6 pg/ml).

VIP and PACAP inhibit TNF-α gene expression in LPS-stimulated microglia

To determine the mechanism by which the $VPAC_1$ receptor agonist inhibits early production of TNF-α, we examined the

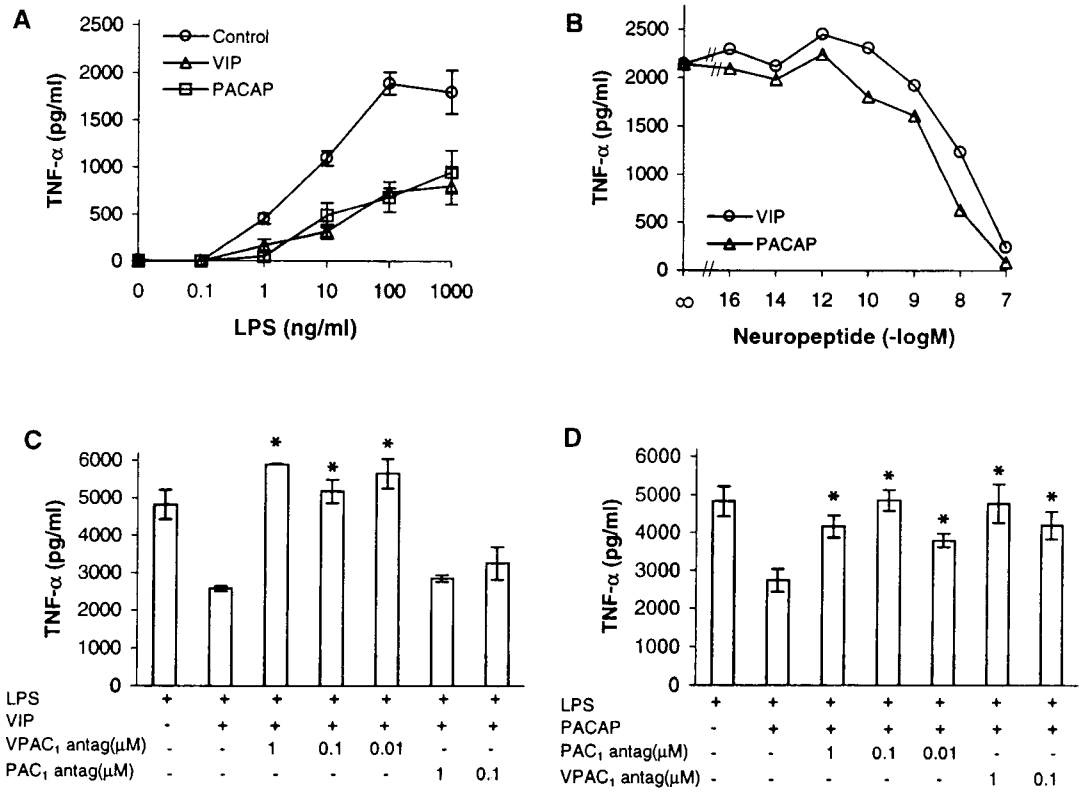

Figure 5. VIP and PACAP inhibit TNF-α protein production via specific receptors. *A*, Microglia were treated for 6 hr with various concentrations of LPS (0.1–1000 ng/ml) in the absence or presence of 10^{-8} M VIP or PACAP. TNF-α accumulation was assayed by ELISA. Each *point* represents the mean ± SEM of TNF-α protein (pg/ml) in three separate cultures. This experiment was repeated with identical results. Data were compared by using an ANOVA with a *post hoc* Fisher's test for comparisons at the 95% confidence level. At all concentrations of LPS above 1 ng/ml, the inhibition produced by VIP and PACAP is significantly different from LPS alone. *B*, Microglia were treated with LPS (100 ng/ml) and various concentrations of VIP or PACAP for 6 hr. Data are expressed as the mean of TNF-α produced from two separate cultures. Microglia were treated with VIP (*C*) or PACAP (*D*) and different concentrations of $VPAC_1$ and PAC_1 antagonists in the presence of LPS (100 ng/ml) for 6 hr. Each *point* represents the mean ± SEM of TNF-α protein (pg/ml) in three separate cultures. This experiment was repeated with similar results. Data were compared by using an ANOVA with a *post hoc* Fisher's test for comparisons at the 95% confidence level. *Different when compared with cultures treated with LPS and VIP or PACAP.

regulation of TNF-α in cultured microglia, the likely cellular source of TNF-α in injured spinal cords (Woodroofe et al., 1991; Lee et al., 1993; Chao et al., 1995; Bartholdi and Schwab, 1997). LPS, a bacterial endotoxin, was used as a stimulant for microglia because it induces an array of inflammatory cytokines (TNF-α, IL-1α, IL-1β, and IL-6) in microglia and macrophages *in vitro* (Lee et al., 1993; Laskin and Pendino, 1995).

A low but detectable level of TNF-α mRNA was present in untreated microglia, but LPS treatment (100 ng/ml) raised levels of TNF-α mRNA substantially as early as 1 hr after exposure (Fig. 2). Simultaneous treatment with VIP (10^{-7} M) inhibited LPS-induced TNF-α mRNA levels 45% (Fig. 3). PACAP (10^{-7} M) completely abolished the LPS-induced increase (Fig. 3). Trypan blue exclusion confirmed that VIP did not affect the viability of microglial cells (data not shown). These data indicate that the LPS-inducible increase in TNF-α gene expression in cultured microglia is inhibited by the authentic neuropeptides VIP and PACAP.

VIP and PACAP downregulate TNF-α protein production in LPS-stimulated microglia

To examine whether inhibition by VIP and PACAP leads to a similar reduction in secreted TNF-α protein, we measured the concentration of TNF-α in the culture supernatants with an ELISA. Consistent with the observations of others (Sawada et al., 1989; Lee et al., 1993; Chao et al., 1995), LPS induced the secretion of TNF-α from enriched microglia cultures in a time-dependent manner (Fig. 4). Although TNF-α production by untreated microglia was undetectable, LPS treatment resulted in the accumulation of 2.6 ng/ml TNF-α after 6 hr. Levels had not returned to baseline values even after 48 hr, the longest time point examined. The effect of LPS was dose-dependent, with maximal TNF-α secretion at 100 ng/ml (Fig. 5*A*). Both VIP and PACAP inhibited LPS-induced increases in TNF-α secretion in a dose-dependent manner, with 90% inhibition at 10^{-7} M (Fig. 5*B*). Even at a submaximal dose (10^{-8} M) both peptides inhibited TNF-α production by microglia stimulated with various LPS concentrations (Fig. 5*A*). Inclusion of a $VPAC_1$ antagonist, but not a PAC_1 antagonist, reversed the VIP reduction in TNF-α production (Fig. 5*C*). On the other hand, inclusion of either antagonist reversed the PACAP reduction in TNF-α production (Fig. 5*D*).

Microglia express $VPAC_1$ and PAC_1 receptors

Both VIP and PACAP use G-protein-linked seven-transmembrane-domain receptors. VIP has highest efficacy at the $VPAC_1$ (or VIP_1) and $VPAC_2$ (VIP_2) receptors, and PACAP has almost equal efficacy at these two receptors as well as a third, the so-called PAC_1 receptor (for review, see Dickinson and Fleetwood-Walker, 1999). To determine which of these receptor

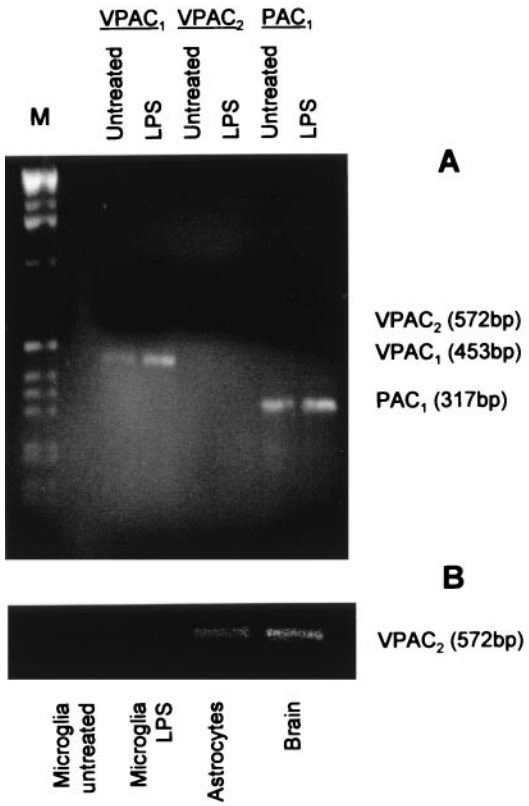

Figure 6. Microglia express mRNAs for $VPAC_1$ and PAC_1 but not $VPAC_2$, receptors. *A*, mRNA from untreated microglia or microglia treated LPS for 3 hr was prepared and examined by RT-PCR (see Materials and Methods). *B*, Rat brain and astrocytic RNA were used as positive controls for $VPAC_2$.

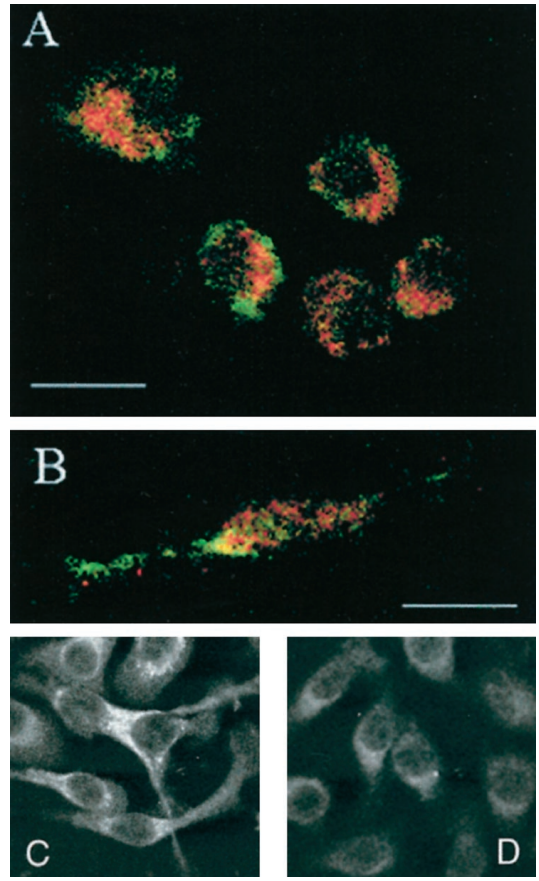

Figure 7. Microglia bind biotinylated VIP. Shown are confocal images of cultured microglia after a 2 hr incubation with LPS (100 ng/ml) together with 10^{-10} M biotinylated VIP (*A*) or after a 1.5 hr incubation with LPS followed by 10^{-10} M biotinylated VIP for 30 min (*B*). To determine binding specificity, we treated HAPI cells for 210 min with LPS. Biotinylated VIP (10^{-10} M) was added for the final 30 min (*C*). Sister cultures were treated the same except for the inclusion of 1 μM unlabeled VIP during the final 150 min of LPS administration (*D*). Confocal images in *C* and *D* were collected by using identical iris, gain, and background settings. Biotinylated VIP was visualized with Texas Red-conjugated avidin D. OX-42 was visualized with FITC-conjugated anti-mouse Ig (see Materials and Methods). Scale bar, 12 μm.

subtypes is responsible for the neuropeptide effect on microglia, we examined mRNA for the three receptors by using RT-PCR analysis. mRNAs for $VPAC_1$ and PAC_1 were expressed in microglia. $VPAC_2$ receptor mRNA was not present even in LPS-treated microglia (Fig. 6).

To demonstrate the presence of VPAC receptors directly, we incubated cultured microglia with LPS (100 ng/ml) and 10^{-10} M biotinylated VIP at 37°C for 2 hr or with LPS for 90 min followed by 10^{-10} M biotinylated VIP for 30 min (Fig. 7*A,B*). The simultaneous immunohistochemical detection of biotinylated VIP (internalized during the long incubation) and OX-42 confirmed the existence of VIP receptors on microglia. The diminished fluorescence intensity obtained with 1 μM unlabeled VIP suggested specificity (Fig. 7*C,D*).

The VIP-induced downregulation of TNF, the presence of $VPAC_1$ and PAC_1 mRNAs, the direct demonstration of VIP binding, and the inhibition by receptor-specific antagonists (see Fig. 5*C,D*) confirm the likelihood of microglia as targets of neuropeptide action.

Neuropeptide action on microglia is mediated by a cAMP-dependent pathway

$VPAC_1$ receptors are coupled exclusively to adenylyl cyclase, whereas PAC_1 receptors are coupled to adenylyl cyclase and/or phospholipase-C, depending on the receptor variant (Dickinson and Fleetwood-Walker, 1999). Because we found effects with both VIP and PACAP, we examined the possible involvement of cAMP, which would be the common downstream effector of both $VPAC_1$ and PAC_1 receptors.

To determine whether cAMP activation mimics neuropeptide action, we included forskolin, an activator of adenylyl cyclase, with LPS (Fig. 8). Forskolin inhibited LPS-induced increases in TNF-α in a dose-dependent manner, confirming that activation of cAMP mimics neuropeptide action. Moreover, H89, a selective PKA inhibitor, reversed the inhibition by VIP of TNF-α protein (Fig. 9) and that of VIP and PACAP on TNF-α mRNA (see Fig. 3). Finally, the inclusion of SNV, a lipophilic VIP analog that does not increase cAMP (Gozes et al., 1995), did not reproduce the inhibitory effect of the neuropeptides (Fig. 10). These data taken together suggest that the neuropeptide inhibition of LPS-induced TNF-α is mediated by $VPAC_1$ and/or PAC_1 receptors via the production of cAMP.

DISCUSSION

Our data have shown that TNF-α mRNA is acutely upregulated after spinal cord transection and that this upregulation is inhibited substantially by a synthetic VIP receptor agonist. Moreover, authentic VIP and the structurally related PACAP are potent inhibitors of LPS-inducible TNF in cultured microglia, the likely

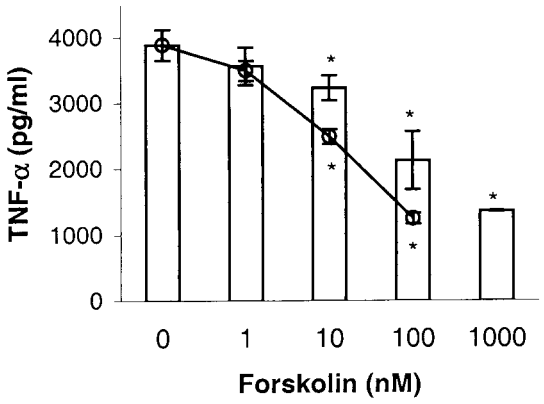

Figure 8. Forskolin mimics the inhibition by neuropeptides of microglial TNF-α production. Microglia were treated with LPS (100 ng/ml) and various concentrations of forskolin (*bars*) or VIP (*curve*). Supernatants were collected after 6 hr and assayed for TNF-α by ELISA. Data are expressed as the mean ± SEM of TNF-α protein in three or four separate cultures and were compared by using an ANOVA with a *post hoc* Fisher's test for 95% confidence levels. *Different when compared with cultures treated with LPS alone.

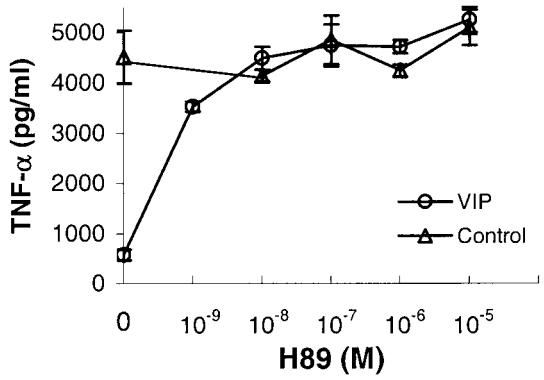

Figure 9. H89 reverses the neuropeptide inhibition of LPS-induced TNF-α production. Microglia were treated with LPS (100 ng/ml) alone (*Control*) or with VIP (10^{-7} M) after a 30 min preincubation with various concentrations of H89. Supernatants were assayed for TNF-α production after 6 hr. Data are expressed as the mean ± SEM of TNF-α protein in three or four separate cultures and were compared by using an ANOVA with a *post hoc* Fisher's test for 95% confidence levels. All concentrations of H89 produced a significant reversal of the VIP-induced inhibition of LPS-induced TNF-α production. Cells treated with H89 alone or H89 plus VIP did not produce detectable amounts of TNF (<15.6 pg/ml).

cellular source of TNF-α in injured spinal cord. The ability of $VPAC_1$ and PAC_1 antagonists to reverse this action, the existence of mRNAs for both receptors, and the direct demonstration of specific VIP binding on cultured microglia all argue strongly for the existence of these neuropeptide receptors on microglia. Three pieces of evidence suggest further that the neuropeptide action is mediated via a cAMP-dependent pathway: (1) the action of the neuropeptides is mimicked by the direct activation of cAMP by forskolin; (2) the neuropeptide-induced inhibition is blocked by H89, a specific inhibitor of PKA; and (3) a neuropeptide analog that does not use a cAMP-mediated pathway fails to reproduce the action of the authentic peptides.

The upregulation of TNF-α is one of the early consequences of CNS insult, but the full spectrum of its action in injured CNS is not clear. Hence, the consequences of inhibiting its production

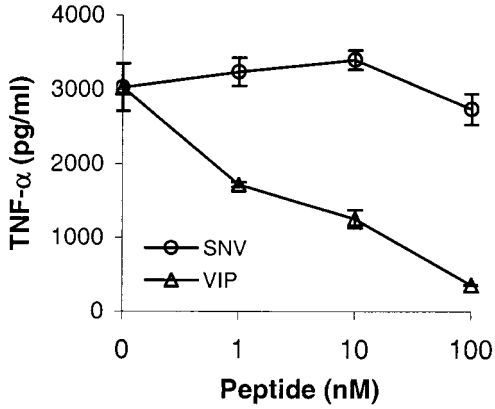

Figure 10. SNV fails to mimic the VIP-induced inhibition of TNF-α production. Cultured microglia were treated with various concentrations of SNV or VIP in the presence of LPS (100 ng/ml). Data are expressed as the mean ± SEM of TNF-α protein in three or four separate cultures and were compared by using an ANOVA with a *post hoc* Fisher's test for 95% confidence levels. Only those cultures with VIP showed a significant inhibition of the LPS-induced TNF-α production.

are unclear. The negative impact of TNF-α is well documented. It causes apoptosis in neuronal cell lines (Talley et al., 1995; Haviv and Stein, 1999) and mediates or potentiates neuronal death induced by LPS (de Bock et al., 1998), trimethyltin (Viviani et al., 1998), HIV Tat protein (New et al., 1998), or glutamate (Chao and Hu, 1994). In models of multiple sclerosis, TNF-α further contributes to CNS damage by killing oligodendrocytes, thereby promoting demyelination (Selmaj and Raine, 1988; Akassoglou et al., 1998). The injured or diseased CNS, then, would seem to benefit from inhibition of its action. This may be particularly true during the early post-traumatic period (Scherbel et al., 1999). The salutary effects of PACAP on cortical cultures compromised by LPS treatment would tend to favor this view (Kong et al., 1999). The rapid and robust upregulation of VIP in the spinal cord (Zhang et al., 1993) and in sympathetic ganglia (Hyatt-Sachs et al., 1993) that follows peripheral axotomy also could be construed as a neuronal protection mechanism designed to damp down the early inflammatory events that accompany such a lesion.

On the other hand, TNF-α also exerts neuroprotective or neurotrophic effects in injured CNS. It protects neurons from death by reactive oxygen species (Cheng et al., 1994; Barger et al., 1995; Mattson et al., 1995; Goodman and Mattson, 1996; Tamatani et al., 1999), possibly by the induction of superoxide dismutase (Sullivan et al., 1999); promotes axonal regeneration (Schwartz et al., 1991); inhibits prolonged inflammation by promoting the production of anti-inflammatory molecules like IL-10 (Sheng et al., 1995); and ultimately helps to reduce CNS tissue loss (Klusman and Schwab, 1997). Mice lacking TNF receptors show increased lesion volumes after traumatic brain injury, further suggesting a protective role for TNF-α (Sullivan et al., 1999).

Effects of TNF-α on brain capillary endothelia provide a clear example of the dichotomous action of TNF. In early stages of inflammation TNF-α promotes blood brain barrier permeability, resulting in damaging edema (Shohami et al., 1996). However, the induction of adhesion molecules on endothelia promotes the migration of leukocytes into a lesioned area (Feuerstein et al., 1994; Vastag et al., 1999). Inhibition of this migration could prove damaging to CNS. Inhibition of TNF, then, would have uncertain

consequences. Thus, determination of the benefits (if any) of neuropeptide treatment awaits studies *in vivo* to assess regenerative outcome.

Our data suggest that the most likely mediator of the neuropeptide-induced TNF inhibition is the cAMP. cAMP-elevating agents inhibit TNF-α biosynthesis in cell types other than microglia (for review, see Jongeneel, 1994), but the intracellular mechanism of this inhibition is not completely understood. Both nuclear factor-κB (NF-κB) and cAMP-responsive element (CRE)-binding complexes function as transactivators in the TNF-α promoter (Yao et al., 1997) and could serve as cAMP/PKA targets. However, studies that used cells of the macrophage/monocyte lineage have shown that increased cAMP levels and/or activated PKA do not affect NF-κB activation and/or mobilization (Albrecht et al., 1995; Ollivier et al., 1996; Delgado et al., 1998), although it has been reported that PKA can bind and phosphorylate the p65 subunit of NF-κB (Zhong et al., 1997, 1998). Although PKA may not affect NF-κB activation directly, CRE-binding proteins (CREB) induced by cAMP could result in changes in the composition of CRE-binding complexes and/or compete with NF-κB for the CREB-binding protein (Parry and Mackman, 1997). Indeed, changes in the composition of the CRE-binding complex have been shown to mediate the cAMP-dependent VIP-induced inhibition of TNF-α production in a macrophage cell line (Delgado et al., 1998). The mechanism underlying the VIP inhibition of TNF production in microglia remains, however, to be determined.

In summary, these experiments show that VIP and PACAP modulate the acute inflammatory response that follows spinal cord injury *in vivo* and microglial activation *in vitro*. Neuropeptide-mediated downregulation of inflammatory cytokines like TNF-α from activated microglia in the CNS may prove valuable as a therapy for CNS injury or disease.

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