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Protein kinase A mediates microglial activation induced by plasminogen and gangliosides

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Abbreviations: CRE, cAMP response element; IL-1β, interleukin-1β; iNOS, inducible nitric oxide synthase; NF-κB, nuclear factor-kappa B; PKA, protein kinase A; TNF-α, tumor necrosis factor-α

Abstract

In the injured brain, microglia is known to be activated and produce proinflammatory mediators such as interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α) and inducible nitric oxide synthase (iNOS). We investigated the role of protein kinase A (PKA) in microglial activation by both plasminogen and gangliosides in rat primary microglia and in the BV2 immortalized murine microglial cell line. Both plasminogen and gangliosides induced IL-1β, TNF-α and iNOS mRNA expression, and that this expression was inhibited by the addition of the PKA inhibitors, KT5720 and H89. Both plasminogen and gangliosides activated PKA and increased the DNA binding activity of the cAMP response element-binding protein (CREB). Furthermore, KT5720 and H89 reduced the DNA binding activities of CREB and NF-κB in plasminogen-treated cells. These results suggest that PKA plays an important role in plasminogen and gangliosides-induced microglial activation.

Keywords: brain inflammation; cAMP response element binding protein (CREB); gangliosides; microglia; plasminogen; protein kinase A

Introduction

Microglia is major immune effector cells in the central nervous system. Microglial activation is a common phenomenon that occurs when the brain is injured (Woodroofe *et al.,* 1986; Giulian *et al.,* 1991). Activated microglia express inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) and the pro-inflammatory cytokines, interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) (Giulian *et al.,* 1986; Zielasek *et al.,* 1992; Lee *et al.,* 1993; Minghetti and Levi, 1995; Pyo *et al.,* 1997; Pyo *et al.,* 1998). Recent studies indicate that microglial activation affects the progression of neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease (Gonzalez-Scarano and Baltuch, 1999; Vila *et al.,* 2001). Such observations highlight the importance of understanding the processes regulating microglial activation.

In the injured brain, microglia can be activated by factors released from damaged cells and also by factors that infiltrate from the blood due to breakdown of the blood brain barrier. The glycospingolipid gangliosides are particularly rich in the neuronal membrane (Derry and Wolfe, 1967), which can be released from damaged neurons into the extracellular space. Previously, we reported that gangliosides induce expression of inflammatory mediators such as TNF-α, iNOS and COX-2 in microglia (Pyo *et al.,* 1999a; Kim *et al.,* 2002). Blood plasma proteins were found to participate in the activation of microglia; thrombin and plasminogen induce expression of inflammatory mediators in microglia (Ryu *et al.,* 2000; Choi *et al.,* 2003; Min *et al.,* 2003).

Many studies have been reported on the signaling pathways involved in microglial activation. Signaling molecules known to mediate activation by compounds such as lipopolysaccharide, gangliosides and thrombin include mitogen-activated protein kinase (MAPK), phospholipase C, protein kinase C and NF-κB (Pyo *et al.,* 1998; Pyo *et al.,* 1999a; Ryu *et al.,* 2000). The role of protein kinase A (PKA) in microglial activation has not been established. In this study, we examined the involvement of PKA in microglial activation induced by plasminogen and gangliosides.

Materials and Methods

Cell culture

Primary microglia were cultured from the cerebral

cortices of 1-3 day-old Sprague Dawley rats as previously described (Giulian *et al.,* 1986; Pyo *et al.,* 1998). Briefly, cortices were triturated into single cells in minimal essential media (Sigma, St. Louise, MO) containing 10% fetal bovine serum (Hyclone, Logan, UT) and seeded into 75 cm^2 T-flasks (0.5 hemisphere/flask). Two weeks later microglia were detached from flasks by mild shaking and applied to a nylon mesh to remove astrocyte and cell clumps. Microglia were seeded into plates or dishes and medium was replaced after one hour in order to remove unattached cells. The BV2 immortalized murine microglial cell line was provided by Dr. E. J. Choi (Korea University). BV2 cells were constructed by infecting primary microglia with a v-raf/v-myc oncogene-carrying retrovirus (J2), and show most morphological, phenotypical and functional properties of freshly isolated microglia (Blasi *et al.,* 1990). BV2 cells were cultured in Dulbecco's Modified Eagle Medium (Gibco, Grand Island, NY) supplemented with 5% fetal bovine serum (Hyclone).

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated using RNAzol^{TM} B (TEL-TEST, Inc., Friendwood, TX), and cDNA was prepared using reverse transcriptase that originated from Avian Myeloblastosis Virus (TaKaRa, Otsu, Japan) according to the manufacturer's instructions. The sequences of PCR primers used for IL-1β, TNF-α, iNOS, GAPDH and actin gene amplifications are shown in Table 1.

Protein kinase A activity assay

Cells were washed twice with cold phosphate-buffered saline, and then lysed in ice-cold lysis buffer (50 mM Tris-HCl, 0.1% Triton X-100) containing protease inhibitors (2 mM phenylmethylsulfonyl fluoride, 100 μg/ml leupeptin, 10 μg/ml pepstatin, 1 μg/ml aprotinin, 2 mM EDTA). Lysates were incubated on ice for 20 min, then centrifuged at 10,000 *g* at 4°C for 20 min, after which the supernatant was collected for assay. The assay mixture contained 5 μg supernatant protein, 50 mM Tris-HCl, 1 mM DTT, 10 mM $MgCl_2$, 30 μM kemptide (PKA substrate, Calbiochem, La Jolla, CA), 25 mM β-glycerophosphate and 2 μCi [γ^{32}P]-ATP. Negative and positive control incubations contained 10 μM PKI (a specific PKA inhibitor) and 5 μM cAMP, respectively. After 5 min incubation, the reaction mixtures were spotted onto phosphocellulose paper, the paper was then washed twice with 1% phosphoric acid and once in 100% ethanol. The radioactivity associated with paper-bound phosphorylated peptide was counted using a liquid scintillation counter.

Measurement of NF-κB and CREB Activation by electrophoresis mobility shift assay (EMSA)

EMSA was carried out as previously described (Pier *et al.,* 1981; Ryu *et al.,* 2000). Briefly, BV2 (5×10^5 cells) were harvested and incubated on ice for 15 min in 900 μl of a hypotonic solution [10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonylfluoride (PMSF)] containing 0.5% nonidet P-40. Cells were centrifuged at 2,000 *g* for 10 min at 4°C and the pellet (nuclear fraction) was collected. The nuclear fraction was resuspended in a buffer containing 20 mM HEPES, pH 7.9, 20% glycerol, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT and 1 mM PMSF, incubated on ice for 60 min with occasional gentle shaking, and then centrifuged at 10,000 *g* for 20 min. The crude nuclear proteins in the supernatant were collected and stored at -70°C until use in EMSA. Synthetic oligonucleotides (Santacruz, California, US) containing the NF-κB binding sequence (5'-AGTTGAGGGGACTTTCCCAGGC-3' and 3'-TCAACTCCCCTGAAAGGGTCCG-5') and the CREB binding sequence (5'-AGAGATTGCCTGACGTCAG-AGAGCTAG-3' and 3'-TATCTAACGGACTGCAGTCT-CTCGATC-5') were end-labeled using Klenow fragment and [γ-^{32}P]-dATP. The labeled DNA probe (approximately 0.2 ng) was incubated for 30 minutes with

Table 1. The sequences of IL-1β, TNF-α iNOS, GAPDH and actin PCR primers.

	Sense	Antisense
Rat IL-1β	5'-TGATGTTCCCATTAGACAGC-3'	5'-GAGGTGCTGATGTACCAGTT-3'
Rat TNF-α	5'-TGATGTTCCCATTAGACAGC-3'	5'-GAGGTGCTGATGTACCAGTT-3'
Rat iNOS	5'-GCAGAATGTGACCATCATGG-3'	5'-ACAACCTTGGTGTTGAAGGC-3'
Rat GAPDH	5'-TCCCTCAAGATTGTCAGCAA-3'	5'-AGATCCACAACGGATACATT-3'
Mouse IL-1β	5'-GCAACTGTTCCTGAACTC-3'	5'-CTCGGAGCCTGTAGTGCA-3'
Mouse TNF-α	5'-ATGAGCACAGAAAGCATGATC-3'	5'-TACAGGCTTGTCACTCGAATT-3'
Mouse iNOS	5'-TCACTGGGACAGCACAGAAT-3'	5'-TGTGTCTGCAGATGTGCTGA-3'
Mouse Actin	5'-CATGTTTGAGACCTTCAACACCCC-3'	5'-GCCATCTCCTGCTCGAAGTCTAG-3'

1 μg nuclear protein in a reaction mixture containing 8.5 mM EDTA, 8.5 mM EGTA, 8% glycerol, 0.1 mM $ZnSO_4$, 50 μg/ml poly (dI-dC), 1 mM DTT, 0.3 mg/ml bovine serum albumin and 6 mM $MgCl_2$. The reaction mixture was separated on an 8% polyacrylamide gel, after which the gel was dried and autoradiographed. To identify specific binding of labeled oligonucleotides to the nuclear protein, excess (×20) unlabeled oligonucleotide was added to control incubations.

Statistical analysis

Data were analyzed using the Statistical Package for Social Sciences 8.0 (SPSS Inc., Chicago, IL).

Results

Protein kinase A (PKA) mediates plasminogen-induced microglial IL-1β, TNF-α and iNOS mRNA expression

Previously, we found that plasminogen induces interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) mRNA expression in microglia (Min *et al.,* 2003). To examine whether PKA mediates microglial activation, we investigated the effect of PKA inhibitors, KT5720 and H89, on plasminogen-induced IL-1β, TNF-α and inducible nitric oxide synthase (iNOS) mRNA expression. We found that in primary cultured microglia, KT5720 and H89 significantly reduced plasminogen-induced IL-1β and TNF-α mRNA expression (Figure 1A). Similar results were obtained when using the BV2 mouse microglial cell line and KT5720 and H89 also reduced plasmignogen-induced iNOS mRNA expression (Figure 1B). Since PKA inhibitors reduced plasminogen-induced IL-1β, TNF-α and iNOS mRNA expression, we investigated whether plasminogen activated PKA. PKA was activated within 10 min of plasminogen addition, and that activity reached a peak after 30 min, after which it decreased (Figure 1C). These results suggest that plasminogen activated microglia via PKA-dependent pathways.

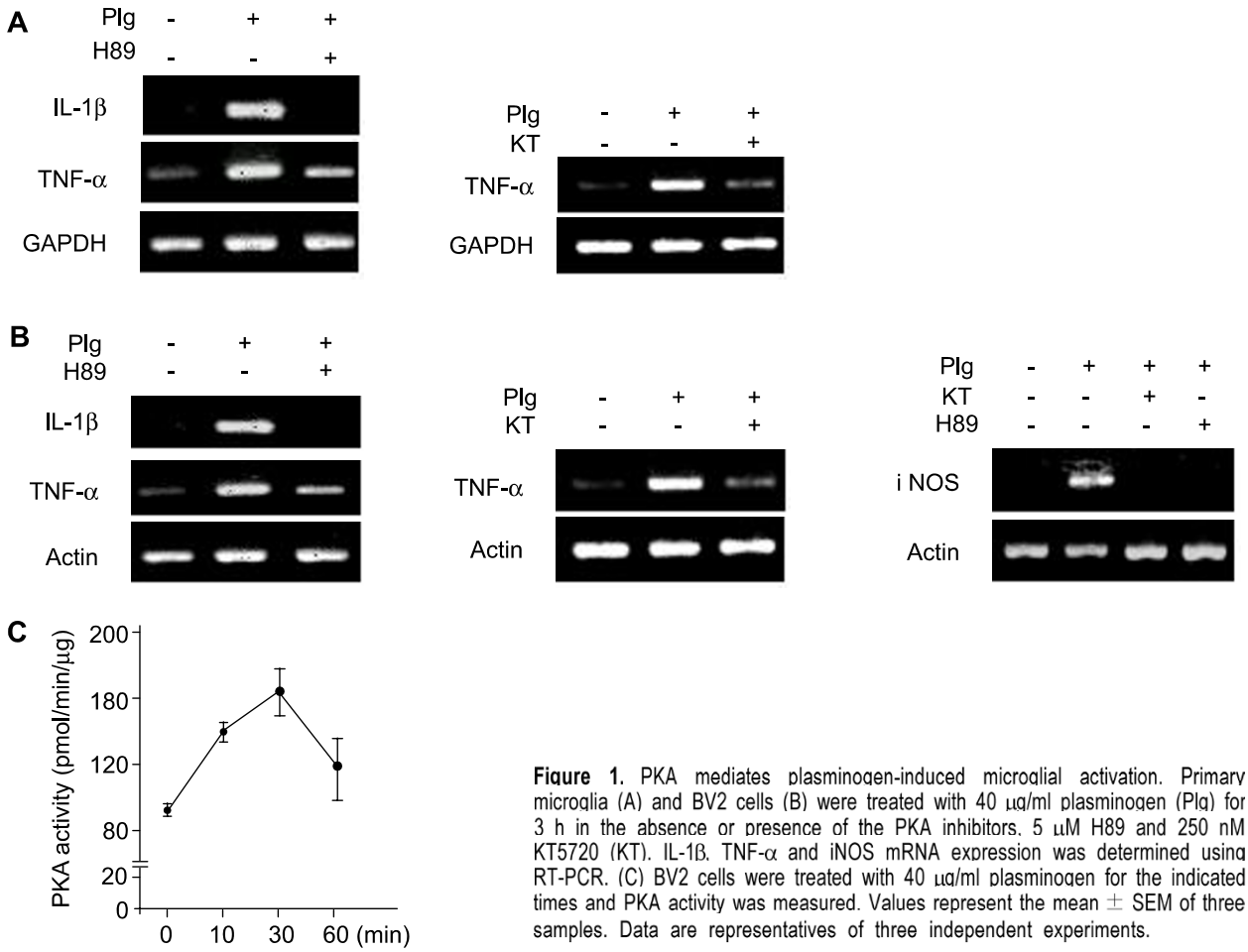

Figure 1. PKA mediates plasminogen-induced microglial activation. Primary microglia (A) and BV2 cells (B) were treated with 40 μg/ml plasminogen (Plg) for 3 h in the absence or presence of the PKA inhibitors, 5 μM H89 and 250 nM KT5720 (KT). IL-1β, TNF-α and iNOS mRNA expression was determined using RT-PCR. (C) BV2 cells were treated with 40 μg/ml plasminogen for the indicated times and PKA activity was measured. Values represent the mean ± SEM of three samples. Data are representatives of three independent experiments.

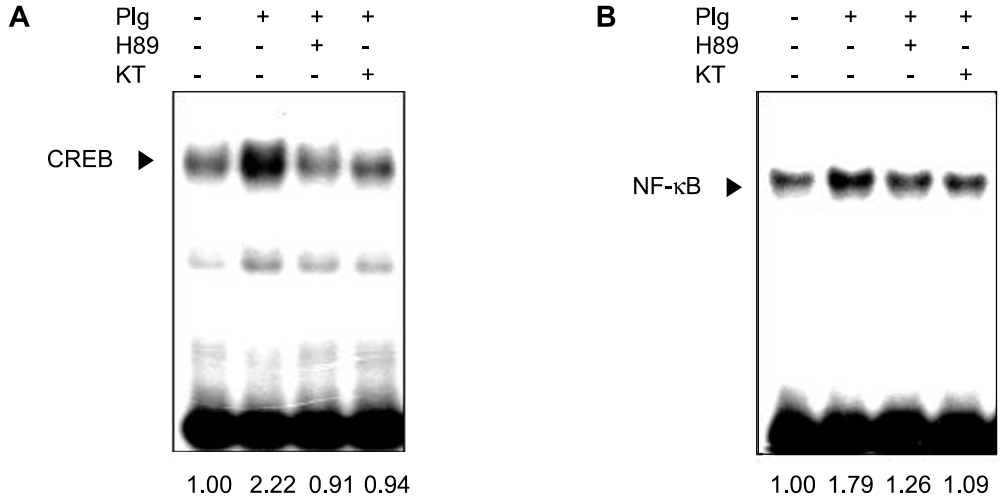

Figure 2. PKA inhibitors reduce the DNA binding activity of CREB and NF-κB in plasminogen-treated microglia. BV2 cells were treated with 40 μg/ml plasminogen (Plg) for 5 min in the absence or presence of the PKA inhibitors, 5 μM H89 or 250 nM KT5720 (KT). Nuclear extracts were prepared and (A) CREB- and (B) NF-κB-specific oligonucleotide-protein complexes were detected using electrophoresis mobility shift assays. The values represent the normalized intensities of the CREB and NF-κB bands against intensities of control bands measured by Image Gauge technology. Data are representatives of three independent experiments.

Plasminogen regulates DNA binding activities of CREB and NF-κB *via* protein kinase A

cAMP response element (CRE) and NF-κB binding sites exist in the promoter regions of genes encoding IL-1β, TNF-α and iNOS (Collart *et al.,* 1990; Hiscott *et al.,* 1993; Wong *et al.,* 1996; Kinugawa *et al.,* 1997; Stylianou and Saklatvala, 1998; Tsai *et al.,* 2000; Chang *et al.,* 2004). Furthermore, plasminogen increases the DNA binding activities of CRE binding protein (CREB) and NF-κB (Min *et al.,* 2003). We examined whether PKA regulated CREB and NF-κB activation. We found that addition of KT5720 and H89 reduced plasminogen-stimulated CREB and NF-κB DNA binding activities (Figure 2). These results suggest that plasminogen increased CREB and NF-κB DNA binding activities through PKA-dependent pathways in microglia.

PKA mediates gangliosides-induced microglial activation

Although we have previously published that gangliosides activate microglia via diverse signaling pathways (Pyo *et al.,* 1999a; Kim *et al.,* 2002; Min *et al.,* in press), the role of PKA in this activation has not been reported. We found that in primary cultured microglia, KT5720 and H89 reduced gangliosides-induced IL-1β, TNF-α and iNOS mRNA expression. (Figure 3A). Furthermore, in BV2 cells, gangliosides activated PKA within 5 min, and activity decreased to basal levels after 30 min (Figure 3B). We also found that gangliosides increased CREB binding activity within 5 min. These results suggest that PKA positively regulates gangliosides-induced microglial activation.

Discussion

Previously, we reported that plasminogen and gangliosides activate microglia (Pyo *et al.,* 1999a; Kim *et al.,* 2002; Min *et al.,* 2003; Min *et al.,* In press). Microglial activation induced by these agents triggers diverse signal transduction pathways such as those involving reactive oxygen species, MAPK, PKC and JAK/STAT (Pyo *et al.,* 1999a; Kim *et al.,* 2002; Min *et al.,* 2003; Min *et al.,* In press). In the present study, we show that the signaling molecule PKA is also involved in positive regulation of microglial activation since plasminogen and gangliosides activated PKA, and PKA inhibitors reduced plasminogen- and gangliosides-induced IL-1β, TNF-α and iNOS mRNA expression.

cAMP is a well-known second messenger that activates PKA. cAMP has been reported to either enhance or reduce iNOS expression depending on the stimuli and cell type. cAMP-elevating agents, including cAMP analogues, norepinephrine and prostaglandins, suppressed LPS-induced iNOS expression in rat microglia (Minghetti *et al.,* 1997), Kupper cells (Mustafa and Olson, 1998) and hepatocytes (Smith *et al.,* 1997). In contrast, cAMP induced iNOS expression in rat vascular smooth muscle cells and mesengial cells (Imai *et al.,* 1994; Eberhardt *et al.,* 1998). In human monocytes, murine astrocytes and rat cardiac myocytes, iNOS expression was induced by beta-endorphin and proinflammatory cytokines that increase

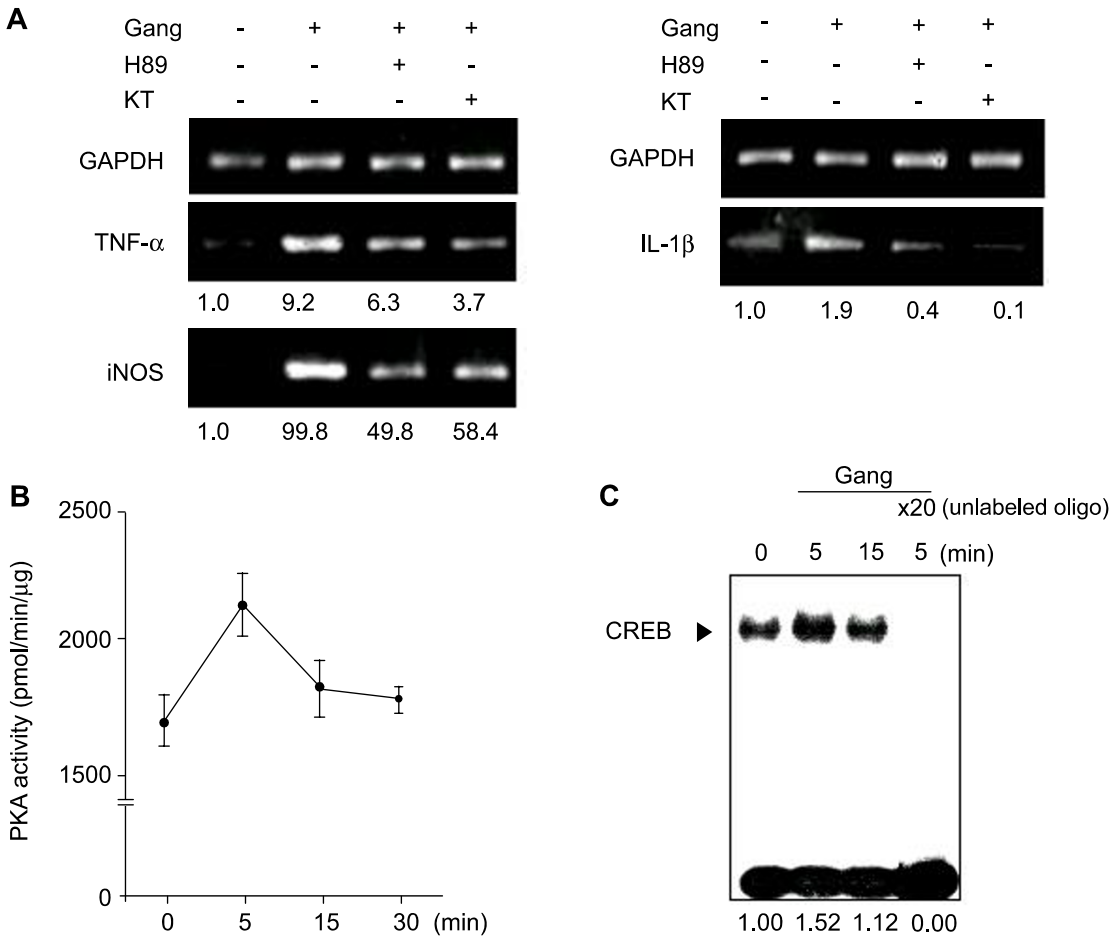

Figure 3. PKA mediates gangliosides-induced microglial activation. (A) Primary cultured microglia were treated with 50 μg/ml gangliosides (Gang) for 3 h in the absence or presence of 5 μM H89 or 250 nM KT5720 (KT). TNF-α, iNOS and IL-1β mRNA expression was determined using RT-PCR. (B) BV2 cells were treated with 50 μg/ml gangliosides for the indicated times and PKA activity measured. Values represent the mean ± SEM of three samples. (C) BV2 cells were treated with 50 μg/ml gangliosides for the indicated times. Nuclear extracts were prepared and CREB-specific oligonucleotide-protein complexes were detected using electrophoresis mobility shift assays. The excess amount of unlabeled oligonucleotide was added to the reaction mixture to determine specific binding of labeled oligonucleotides to the nuclear protein (×20). The values under the figures represent the normalized intensities of the TNF-α, iNOS and IL-1β bands against intensities of GAPDH bands (A) or normalized intensities of CREB bands against intensity of the control band (C) measured by Image Gauge technology. Data are representatives of three independent experiments.

intracellular cAMP levels (Oddis *et al.,* 1996; Burgher *et al.,* 1997; Aymerich *et al.,* 1998). In microglia, we found that cAMP-elevating agents such as cholera toxin and dbcAMP enhanced beta-amyloid-(Pyo *et al.,* 1999b) and LPS-induced NO release (data not shown). This differential effect of cAMP on iNOS expression may reflect different cell types and/or different stimulators. Furthermore, the duration and extent of intracellular cAMP increase and PKA activity may differentially regulate iNOS expression, even in the same cell type (Galea and Feinstein, 1999).

An increase in intracellular cAMP can induce the expression of many genes via PKA-mediated phosphorylation of transcription factors, such as the cAMP response element binding protein (CREB) (Chandra *et al.,* 1995). The promoter regions of genes encoding IL-1β, TNF-α and iNOS contain CREB binding sites (Stylianou and Saklatvala 1998; Galea and Feinstein, 1999; Tsai *et al.,* 2000). Therefore, PKA may regulate IL-1β, TNF-α and iNOS expression via CREB phosphorylation. In a previous study and this study, we showed that plasminogen and gangliosides enhanced CREB DNA binding activity (Figure 3C, Min *et al.,* 2003). Furthermore, PKA inhibitors reduced plasminogen-induced CREB binding activity (Figure 2A). Thus, we propose that CREB is a PKA target during plasminogen- and gangliosides-induced microglial activation.

NF-κB binding sites exist in the promoter regions of IL-1β, TNF-α and iNOS genes (Collart *et al.,* 1990;

Hiscott *et al.,* 1993; Xie *et al.,* 1993; Chang *et al.,* 2004). NF-κB activation may be regulated by PKA since in J774 macrophage-like cells, H89 inhibited LPS-induced NF-κB activation (Muroi and Suzuki, 1993). The results presented in the current study show that PKA inhibitors reduced NF-κB DNA binding activity in plasminogen-treated microglia (Figure 2B).

Extracellular-signal regulated kinase (ERK) may be another target of PKA. In PC12 cells, PKA is required for sustained activation of ERK (York *et al.,* 1998). We previously reported that ERK mediates gangliosides-induced microglial activation since gangliosides activated ERK, and the ERK pathway inhibitor, PD98059, reduced gangliosides-induced NO release (Pyo *et al.,* 1999a). In the present study, we found that both KT5720 and H89 reduced ERK activation in gangliosides-treated microglia (data not shown). In conclusion, the current results suggest that PKA is a positive regulator of microglial activation. Furthermore, the data suggest PKA exerts this control by acting as an upstream regulator of CREB and NF-κB.

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