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ICAM-1-Coupled Signaling Pathways in Astrocytes Converge to Cyclic AMP Response Element-Binding Protein Phosphorylation and TNF-α Secretion

Sandrine Etienne-Manneville, Nathalie Chaverot, A. Donny Strosberg, and Pierre-Olivier Couraud

In the CNS, astrocytes play a key role in immunological and inflammatory responses through ICAM-1 expression, cytokine secretion (including TNF-α), and regulation of blood-brain barrier permeability. Because ICAM-1 transduces intracellular signals in lymphocytes and endothelial cells, we investigated in the present study ICAM-1-coupled signaling pathways in astrocytes. Using rat astrocytes in culture, we report that ICAM-1 binding by specific Abs induces TNF-α secretion and activation of the transcription factor cAMP response element-binding protein. We show that ICAM-1 binding induces cAMP accumulation and phosphorylation of the mitogen-activated protein kinase extracellular signal-regulated kinase. Both pathways are responsible for cAMP response element-binding protein phosphorylation and TNF-α secretion. Moreover, these responses are partially dependent on protein kinase C, which acts indirectly, as a common activator of cAMP/protein kinase A and extracellular signal-regulated kinase pathways. These results constitute the first evidence of ICAM-1 coupling to intracellular signaling pathways in glial cells and demonstrate the convergence of these pathways onto transcription factor regulation and TNF-α secretion. They strongly suggest that ICAM-1-dependent cellular adhesion to astrocytes could contribute to the inflammatory processes observed during leukocyte infiltration in the CNS. The Journal of Immunology, 1999, 163: 668–674.

Following pathological injuries, trafficking of leukocytes into tissues is a key feature of the host systemic immune response. In the CNS, despite the presence of the blood-brain barrier, immunological response to pathological situations, such as multiple sclerosis or viral infections, is also mediated by infiltration of activated leukocytes into the brain parenchyma and their interaction with resident cells.

Among the numerous cell surface adhesion molecules involved in this process, ICAM-1 (CD54) and its ligands LFA-1 and Mac-1 constitute key players in leukocyte adhesion, extravasation, and Ag presentation (for review, see Ref. 1). CNS cells such as glial cells, brain endothelial cells, and neurons can express ICAM-1 in vitro (2, 3). ICAM-1 expression is frequently up-regulated in inflammatory diseases of the CNS. It is strongly detected in a subset of reactive astrocytes surrounding seniles plaques in brain tissue from Alzheimer’s disease patients (4) and in brain tissue from multiple sclerosis patients, as well as from animals with experimental allergic encephalomyelitis, the animal model for multiple sclerosis (5, 6). In vitro, ICAM-1 expression on glial cells can be up-regulated in response to cytokines such as TNF-α, IL-1β, and IFN-γ (7), or following measles virus infection or HIV gp120 expression (8, 9). Adhesion and migration of leukocytes to inflammatory sites in the brain seem to require ICAM-1 (10, 11), which also largely contributes to the adhesion of polymorphonuclear cells and monocytes on CNS cells (2, 5).

Engagement of ICAM-1 with its ligands induces signals across the plasma membrane to the cytoplasm. In brain endothelial cells, ICAM-1 cross-linking leads to actin-based cytoskeleton rearrangements, stimulation of Src kinase activity, and tyrosine phosphorylation of cytoskeleton-associated protein (3, 12, 13). In lymphocytes, ICAM-1 stimulation leads to B cell receptor signaling, tyrosine phosphorylation, and cytokine release (14–16). Although ICAM-1 expression in the CNS has been reported, the physiological consequences of ICAM-1 stimulation in glial cells have not been explored yet.

Astrocytes are the most numerous glial cells in the CNS and are critical for maintaining a homeostatic environment for neurons. Moreover, perivascular astrocytes regulate the integrity of the blood-brain barrier by controlling the formation of tight junctions between brain endothelial cells (17). The additional function ascribed to astrocytes as APCs suggests a central role for astrocytes in the development of immune responses within the CNS. Together with microglia, they are generally considered as the major source of cytokines in the CNS (for review, see Ref. 18). In vitro, astrocytes secrete a variety of cytokines such as TNF-α, in response to various biological stimuli. Although TNF-α may have neuroprotective activity (19), glial response to TNF-α is usually neurotoxic (20). TNF-α is highly secreted during neuroinflammatory diseases such as bacterial meningitis (21), cerebral malaria (22), and multiple sclerosis (23). It can promote infiltration of inflammatory cells into the CNS, cytokine production, cytotoxicity to oligodendrocytes, and astrogliosis (18, 24), although its pathogenic role in multiple sclerosis is still debated (25, 26).
It has been reported that activated lymphocytes can bind to astrocytes (27), and interestingly, the level of endogenous TNF-α greatly increases in response to monocyte adhesion on CNS cells (2). These observations led us to determine, using primary cultures of rat astrocytes, whether ICAM-1 binding could induce intracellular signals involved in TNF-α secretion.

Materials and Methods

Cell culture

Primary cultures of astrocytes were prepared as previously described (28). Striata were dissected out from brains of 17-day-old CD rat embryos and were dissociated mechanically in serum-free medium. Cells were plated on (poly)l-ornithine (1.5 μg/ml)-precoated dishes of 60 mm diameter, in DMEM containing 1 g/L glucose, supplemented with 10% FCS, and 10 mM HEPES, pH 7.4. After 21 days in culture at 37°C and 5% CO₂, routinely more than 95% of the cells were positively stained for glial fibillary acidic protein (Amersham, Arlington Heights, IL) and for ICAM-1. Cultures were further enriched in astrocytes by washing in calcium- and magnesium-free PBS, by trypsinization (0.25% trypsin, 0.02% EDTA) and subsequent seeding on (poly)l-ornithine (1.5 μg/ml)-precoated dishes (29). Treatments were performed 10 days later on confluent cells. Cell viability was checked by trypan blue exclusion following the various treatments or pretreatments.

Reagents

Mouse mAb to rat ICAM-1 (1A29) was purchased from Serotec (Wiesbaden, Germany). Rabbit anti-mouse Abs were from Dako (Trappes, France). Polyclonal Abs directed against CREB1 and Ser133-phosphorylated CREB were from UBI (Lake Placid, NY). mAb anti-Erk-2, used for Western blotting, was from Santa Cruz Biotechnology (Santa Cruz, CA). Escherichia coli LPS, calcium ionophore A23187, PMA, and forskolin were from Sigma (St. Louis, MO). The highly selective cAMP-dependent protein kinase; PKC, protein kinase C; Erk, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; PKA, cAMP-dependent protein kinase; PKC, protein kinase C.-

TNF-α assays

Astrocytes (10⁶ cells) were placed at confluence in serum-free medium for 24 h and washed with PBS before treatment. After 3 days (or as indicated), cells were collected and TNF-α was measured by ELISA (Amersham, Buckinghamshire, U.K.). TNF-α bioactivity was determined by cytotoxicity assay using actinomycin D-treated L929 cells (30). Briefly, L929 cells (2.5 × 10⁴ cells/well) were seeded in 96-well plates, and incubated for 24 h and subsequently 24 h with 400 ng/well of actinomycin D (Sigma), and 100 μl of serial dilutions of samples or rTNF-α (Calbiochem). Dead cells were washed with PBS and viable cells were stained by addition of 10,000 × g for 10 min. Lysates were incubated overnight at 4°C with anti-Erk-1 or anti-Erk-2 polyclonal Abs, with protein A-agarose. Immunoprecipitates were collected by centrifugation and extensively washed in Nonidet-P40 buffer. The last wash was done with kinase buffer (20 mM HEPES, pH 7.4, 10 mM MgCl₂, 1 mM DTT, 10 mM paranitrophenylphosphate). Reactions were initiated by resuspending the beads in a total volume of 40 μl of kinase buffer containing 0.25 mg/ml myelin basic protein, 40 μM ATP, and 2.5 μCi [γ-32P]ATP. After 10 min shaking at 30°C, reactions were stopped by addition of 10 μl of formic acid. After a brief centrifugation, 35 μl of the supernatant fraction was spotted onto Whatman P81 chromatography paper squares, and unincorporated [γ-32P]ATP was eluted by washing in 175 mM phosphoric acid. Radioactivity incorporated into myelin basic protein was measured by β-scintillation spectrophotometry.

SDS-PAGE and Western blot analysis

Astrocytes were incubated with serum-free medium for 24 h. Cells were washed in PBS before treatments. Following treatments, astrocytes were washed with ice-cold PBS and lysed with SDS sample buffer (100 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 1 mM orthovanadate, 100 mM DTT with bromophenol blue). Cellular extracts were then loaded on polyacrylamide gel and submitted to electrophoresis, as previously described (12). For serial incubations of membranes, bound Abs were stripped out by treatment with higher concentrations of anti-ICAM-1 mAb (Fig. 1A, left panel) or irrelevant rabbit Abs. After washes, the cells were incubated for 1 h with Cy3-conjugated goat anti-rabbit Abs. Immunofluorescence images were collected in a scanner confocal microscope (MCR.1000; Bio-Rad, Hercules, CA).

Results

ICAM-1 binding on rat astrocytes induces TNF-α secretion

Cultured rat astrocytes were incubated for 24 h in serum-free medium, then treated for 72 h with anti-ICAM-1 (1A29) mAb. As shown in Fig. 1A, nontreated astrocytes (NT) did not produce TNF-α constitutively, but could be stimulated by anti-ICAM-1 mAb to secrete TNF-α, to a level corresponding to about one-third of that released by astrocytes treated with 100 ng/ml of LPS. Cross-linking of anti-ICAM-1 mAb with rabbit anti-mouse Abs did not lead to a significantly higher TNF-α secretion (data not shown), suggesting that ICAM-1 aggregation was not required for this response. Treatment with irrelevant isotype-matched mAb did not induce any detectable TNF-α secretion (Fig. 1A, left panel). Biological activity of immunoreactive TNF-α, quantified by cytotoxicity assay using actinomycin D-treated L929 fibroblasts (Fig. 1A, right panel), confirmed the ratio of TNF-α levels upon ICAM-1 binding and LPS treatment.

Kinetics analysis indicated that anti-ICAM-1 mAb (Fig. 1C) leads to an increasing accumulation of TNF-α in cell supernatants between 24 and 72 h (Fig. 1B, left panel). TNF-α production by anti-ICAM-1-stimulated astrocytes was dose dependent in a range of 2.5–10 μg/ml mAb concentrations (Fig. 1B, right panel). Treatment with higher concentrations of anti-ICAM-1 mAb did not give rise to a significantly higher TNF-α production.

ICAM-1 binding triggers cAMP accumulation and Erk activation

TNF-α secretion by astrocytes is known to be due to induction of TNF-α gene transcription (29, 31). Investigating ICAM-1-coupled signaling pathways upstream of TNF-α secretion, we focused our study on the following major regulators of transcription factors: cAMP-dependent protein kinase (PKA), protein kinase C (PKC), and the mitogen-activated protein kinases Erk and JNK.
Treatment with anti-ICAM-1 mAb, but not with an irrelevant isotype-matched (IgG1) Ab, induced cAMP accumulation in astrocytes (Fig. 2A). This observation was confirmed by using another anti-ICAM-1 mAb (3H8; not shown). ICAM-1-mediated cAMP accumulation was dose dependent in a range of 1–20 μg/ml of mAb (Fig. 2B).

Erk phosphorylation, which has been shown to correlate with activation, was assessed by demonstrating reduced electrophoretic mobility of phosphorylated Erk-2 isoform and Raf, its upstream activator (Fig. 3A). Indeed, treatment with anti-ICAM-1 mAb for 20 min induced Erk-2 and Raf phosphorylation. As above, this response was also observed with another anti-ICAM-1 mAb (not shown) and not in response to an isotype-matched (IgG1) Ab. Furthermore, Erk-2 as well as Erk-1 activation were confirmed by direct activity assay after specific immunoprecipitation (Fig. 3B).

Kinetics of the response indicates that Erk-1 and Erk-2 activity increased rapidly following treatment with anti-ICAM-1 mAb, reaching a maximum at respectively 10 and 20 min, then decreasing to basal levels during the following hour (Fig. 3B, left panel). As shown in Fig. 3B (right panel), these responses were dose dependent and reached a plateau at 20 and 40 μg/ml of anti-ICAM-1 mAb. No change in Erk-1 or Erk-2 activity was observed after treatment with an irrelevant isotype-matched Ab (not shown).

In contrast with our previous observations in brain endothelial cells (12), we could not detect any increase in JNK activity following ICAM-1 engagement in astrocytes (data not shown), ruling out a role for JNK in TNF-α production induced by ICAM-1.

Taken together, these results indicate that ICAM-1 engagement in astrocytes induces activation of PKA and Erk pathways, suggesting that these pathways might lead to TNF-α secretion.

PKC involvement in ICAM-1-coupled signaling pathways

Because PKCs can activate Erk cascade and cAMP synthesis in different cell types (32, 33), we investigated a putative role of PKCs in ICAM-1-coupled cAMP/PKA and Erk pathways.
ICAM-1 engagement stimulates CREB phosphorylation at Ser\textsuperscript{133}

The transcription factor, CREB, is directly regulated by PKA (34), and has also been shown more recently to be regulated by other signaling pathways, including PKCs and Erk (35). Activation of these pathways following ICAM-1 binding led us to study the regulation of this transcription factor in astrocytes. The ability of CREB to activate transcription is known to be regulated by phosphorylation at residue Ser\textsuperscript{133} (34). Using Abs that specifically recognize this Ser\textsuperscript{133}-phosphorylated form of CREB, we observed that CREB phosphorylation and TNF-\textalpha production observed following ICAM-1 engagement in astrocytes, we monitored these responses in the presence of specific inhibitors: H89, PD98059, and overnight PM pretreatment (or GF 109203X), respectively.

Pretreatment with H89 almost totally abolished CREB phosphorylation induced by the cAMP-elevating agent forskolin, as expected, but only partially prevented the effect of anti-ICAM-1 Abs (Fig. 6A). In parallel, H89 pretreatment completely blocked forskolin-induced TNF-\textalpha secretion, and significantly inhibited both Erk and cAMP/PKA signaling pathways induced by ICAM-1 engagement in astrocytes.

We observed that 10-min PMA treatment of astrocytes, which is known to activate most PKC isoforms, led to Erk-2 activation and Raf phosphorylation, as indicated by the electrophoretic mobility shift of these proteins (Fig. 4A, right panels). Depletion of PKC-sensitive isoforms by overnight pretreatment with PMA totally abolished these responses, as expected, and largely reduced ICAM-1-coupled Raf and Erk-2 phosphorylation. Inhibition of Erk-1 and Erk-2 activation was confirmed by activity assay (data not shown).

PKC stimulation by 10-min PMA treatment also elevated cAMP level (Fig. 4B); this effect being abolished was by overnight pretreatment with PMA. In the same conditions, cAMP accumulation, observed after ICAM-1 binding, was reduced by about 50%.

These results show that PKCs are involved in the activation of both Erk and cAMP/PKA signaling pathways induced by ICAM-1 engagement in astrocytes.

**FIGURE 4.** Role of PKC in Raf/Erk and cAMP/PKA pathways in astrocytes. Cells were either nontreated (NT) or stimulated with anti-ICAM-1 mAb (1A29, 10 \( \mu \)g/ml) for 20 min or treated with PMA (160 nM) for 10 min in serum-free medium; when indicated (PMA O/N (+)), cells have been pretreated with PMA (160 nM, 14 h) for PKC depletion. A, Cell extracts were analyzed by SDS-PAGE and submitted to Western blotting with anti-Raf and Erk-2 Abs. In the same conditions, cAMP accumulation assay was performed as described in Materials and Methods. Results are means \( \pm \) SD of three experiments. Results are representative of four independent experiments.

**FIGURE 5.** Induction of CREB phosphorylation following ICAM-1 engagement on astrocytes. A, Cells were either nontreated (NT) or stimulated with anti-ICAM-1 mAb (1A29, 10 \( \mu \)g/ml) or isotype-matched mAb (IgG1, 10 \( \mu \)g/ml) in serum-free medium for 20 min. B, Cells were stimulated with anti-ICAM-1 mAb (1A29, 10 \( \mu \)g/ml) for the indicated periods of time. Cell extracts were analyzed by SDS-PAGE and submitted to Western blotting with anti-Ser\textsuperscript{133}-phosphorylated CREB (anti-P-CREB, upper panel). As control for protein loading, Western blots with anti-CREB Abs, which do not discriminate between phosphorylated and nonphosphorylated forms of CREB, were performed on the same membrane (anti-CREB, lower panel). C, Cells were either nontreated (NT) or stimulated with anti-ICAM-1 mAb (1A29, 10 \( \mu \)g/ml) in serum-free medium for 30 min, then they were labeled using anti-Ser\textsuperscript{133}-phosphorylated CREB (anti-P-CREB) plus Cy3-conjugated anti-mouse Abs. Results presented are representative of three to five independent experiments.
TNF-α F14 h) ( ). Hatched bars indicate that cells have not been pretreated (tracts were analyzed by SDS-PAGE and submitted to Western blotting), specific inhibitors, respectively, of PKA, MEK, and PKCs. Cell ex- affect TNF-α induced by ICAM-1 binding. H89 by itself did not significantly levels (Fig. 6 E). After stripping, Western blotting was performed with anti-Erk-2 Abs (upper panel). Results are representative of four independent experiments. B, D, and F. Cells were either nontreated (NT) or stimulated with anti-ICAM-1 mAb (1A29, 10 μg/ml) or forskolin (FK, 10 −3 M), for 72 h in serum-free medium. Before stimulation, astrocytes were pretreated (grey bars) with: H89 (20 μM, 1 h) (A); PD 98059 (10 μM, 30 min) (C); PMA (160 nM, 14 h) or GF109203X (4 μg/ml, 1 h) (E), specific inhibitors, respectively, of PKA, MEK, and PKCs. Cell extracts were analyzed by SDS-PAGE and submitted to Western blotting with anti-Ser133-phosphorylated CREB (anti-P-CREB). C. After stripping, Western blotting was performed with anti-Erk-2 Abs (C, upper panel). Results are representative of four independent experiments. B, D, and F. Cells were either nontreated (NT) or stimulated with anti-ICAM-1 mAb (1A29, 10 μg/ml) or forskolin (FK, 10 −3 M), for 72 h in serum-free medium. Before stimulation, astrocytes were pretreated (grey bars) with: H89 (20 μM, 1 h) (B); PD 98059 (10 μM, 30 min) (D); or PMA (160 nM, 14 h) (F). Hatched bars indicate that cells have not been pretreated ( ). TNF-α in cell supernatants was quantified by ELISA. Results are the means ± SD of three independent determinations.

FIGURE 6. Determination of signaling pathways involved in ICAM-1-coupled TNF-α secretion. A, C, and E. Rat astrocytes were either nontreated (NT) or stimulated with anti-ICAM-1 mAb (1A29, 10 μg/ml) or forskolin (FK, 10 −3 M) for 20 min in serum-free medium. Before stimulation, astrocytes were pretreated with: H89 (20 μM, 1 h) (A); PD 98059 (10 μM, 30 min) (C); PMA (160 nM, 14 h) or GF109203X (4 μg/ml, 1 h) (E), specific inhibitors, respectively, of PKA, MEK, and PKCs. Cell extracts were analyzed by SDS-PAGE and submitted to Western blotting with anti-Ser133-phosphorylated CREB (anti-P-CREB). C. After stripping, Western blotting was performed with anti-Erk-2 Abs (C, upper panel). Results are representative of four independent experiments. B, D, and F. Cells were either nontreated (NT) or stimulated with anti-ICAM-1 mAb (1A29, 10 μg/ml) or forskolin (FK, 10 −3 M), for 72 h in serum-free medium. Before stimulation, astrocytes were pretreated (grey bars) with: H89 (20 μM, 1 h) (B); PD 98059 (10 μM, 30 min) (D); or PMA (160 nM, 14 h) (F). Hatched bars indicate that cells have not been pretreated ( ). TNF-α in cell supernatants was quantified by ELISA. Results are the means ± SD of three independent determinations.

night PMA pretreatment. In the same conditions of pretreatment, anti-ICAM-1-induced CREB phosphorylation was only partially inhibited (Fig. 6E, upper panel). Similar results were observed using the PKC inhibitor, GF 109203X (37) (Fig. 6E, lower panel). Overnight PMA pretreatment also caused a partial inhibition of anti-ICAM-1-induced TNF-α secretion (49% inhibition, Fig. 6F).

Taken together, these results indicate that all three kinases, PKA, PKC, and Erk, contribute to CREB phosphorylation as well as TNF-α secretion in response to ICAM-1 binding. This observation raises the question of the relative contribution of each of these pathways.

Relative contribution of ICAM-1-coupled signaling pathways to CREB phosphorylation

Because PKCs can directly phosphorylate CREB in vitro (38), we investigated whether PMA-induced CREB phosphorylation in astrocytes was direct or mediated by cAMP/PKA and Erk pathways. Inhibition of Erk or PKA pathways by PD98059 and H89, respectively, reduced PMA-induced CREB phosphorylation to similar levels (Fig. 7A). When both pathways were blocked, inhibition was complete, strongly suggesting that, in astrocytes, 1) PKCs cannot phosphorylate CREB directly, and that 2) PMA-induced CREB phosphorylation is mainly mediated by cAMP/PKA and Erk pathways (Fig. 8).

CREB phosphorylation induced by ICAM-1 binding was also similarly reduced by H89 or PD98059 to similar levels (Fig. 7B). Depletion of PKCs caused a stronger inhibition, confirming that Erk and PKA pathways are largely PKC dependent (Fig. 7B). Indeed, following cell pretreatment with H89 and PMA or with PD98059 and PMA, a slight phosphorylation of CREB was still detectable (Fig. 7B), most likely reflecting the existence of minor

(63%) inhibition, but not completely, reduced TNF-α secretion induced by ICAM-1 binding. H89 by itself did not significantly affect TNF-α level (Fig. 6B).

Pretreatment with PD98059, a selective inhibitor of the kinase MEK, which phosphorylates and activates Erk-2 (36), totally inhibited Erk-2 activation, as expected (Fig. 6C, upper panels). This pretreatment partially reduced CREB phosphorylation in response to ICAM-1 engagement, not to forskolin (Fig. 6C, lower panel). Interestingly, forskolin (as well as H89 pretreatment; data not shown) did not induce any change in Erk-2 activity, indicating that cAMP accumulation is not a mediator of Erk-2 activation in astrocytes. In parallel, TNF-α secretion observed in response to ICAM-1 binding was also largely inhibited by PD98059 pretreatment (75% inhibition, Fig. 6D).

Treatment of astrocytes with PMA, for 10 min, induced CREB phosphorylation (Fig. 6E), which was totally abolished by over-
PKC-independent Erk and PKA pathways, respectively (Fig. 8). Similar observations were obtained by using GF109203X to inhibit PKCs (data not shown). Finally, concomitant treatment with H89 and PD98059 completely blocked anti-ICAM-1-induced CREB phosphorylation (Fig. 7B), pointing to PKA and Erk pathways as the major direct mediators of CREB phosphorylation in response to ICAM-1 engagement in astrocytes (Fig. 8).

Discussion

We have shown in this study that ICAM-1 engagement on primary astrocytes leads to TNF-α secretion together with phosphorylation of the transcription factor CREB, and we have characterized intracellular pathways involved in both responses. Our results highlighted a central role of PKCs as key mediators of ICAM-1-coupled activation of cAMP/PKA and Erk pathways. Moreover, we have shown that these two pathways converge onto CREB phosphorylation (Fig. 8) and participate in the induction of TNF-α secretion.

Interestingly, ICAM-1-coupled signaling pathways in astrocytes appear different from those observed in brain endothelial cells, reflecting cellular specificity. Indeed, ICAM-1-coupled cAMP accumulation reported in this study does not occur in brain endothelial cells and, to our knowledge, has not been reported in immune cells. Indeed, PKC-dependent accumulation of cAMP most likely reflects the expression, in astrocytes, of brain-specific adenyl cyclase type II, which is known to be regulated by PKC.

Although raises of cAMP intracellular levels were initially described to specifically activate CREB (34), CREB was found subsequently to be phosphorylated, at Ser133, in response to calcium influx or growth-factor stimulation (39, 40). We describe in this study that CREB can also be involved in response to cell-cell adhesion. It is likely that upon ICAM-1 binding, Erk leads to phosphorylation of CREB through activation of its effectors, ribosomal S6 kinases, known to phosphorylate CREB at Ser133 in vitro and in vivo upon growth-factor stimulation (41). In addition, other Erk-activated CREB kinases may also exist in astrocytes, as reported in melanocytes (42). The molecular mechanisms responsible for PKC-mediated CREB phosphorylation are still unclear. Although PKCs can phosphorylate CREB in vitro (38), our results strongly suggest that PKC-induced CREB phosphorylation is not direct, but mediated by two major pathways, Erk cascade and cAMP/PKA. Studies on calcium signal transduction in PC12 cells and glial cell progenitors led to the proposal that calmodulin kinases may be the calcium-activated enzyme that phosphorylates CREB (40). However, we observed in this study that calcium ionophore treatment did not induce CREB phosphorylation, suggesting that calcium influx per se is not a key mediator of CREB phosphorylation in astrocytes (not shown). In addition, although the pathways described in the present study converge to the regulation of the transcription factor CREB, it is likely that they regulate other transcription factors, such as Elk-1, known to be phosphorylated following Erk activation (43), or AP-1, a responsive element for PKCs, which may also contribute to transcription regulation.

CREB phosphorylation at Ser133 is usually followed by transcriptional activation of CRE-dependent genes (40, 41). Different mechanisms underlie this process, which may involve binding of phosphorylated CREB to a CRE-binding protein, followed by interaction of this complex with the transcriptional machinery (44). Once it is phosphorylated in growth factor-stimulated cells, CREB plays a critical role in the activation of immediate early genes. Indeed, in response to nerve growth factor, CREB contributes to the activation of c-fos transcription (39) and is required for the full response of zif268 and nur77 (45, 46). The protein products of these immediate early genes may then activate the transcription of late response genes, such as matrix metalloproteases or cytokines, such as TNF-α. In addition, because CREB binding sites have been found within the promoter of a number of these late response genes, CREB can also directly regulate their expression (47–49). Our results show that the same proteins were involved in both CREB phosphorylation and TNF-α production, suggesting that anti-ICAM-1-induced TNF-α secretion in astrocytes depends on CREB phosphorylation. Indeed, CREB activation has been suggested to be sufficient for enhancing the transcription of TNF-α (50).

In a normal situation, TNF-α is not expressed in astrocytes, but TNF-α gene is transcriptionally activated upon exposure to multiple stimuli (29, 31, 31). Astrocyte overexpression of TNF-α is considered a characteristic feature of inflammatory diseases in the CNS. Extrapolation of our data suggests that leukocyte-astrocyte interaction, via ICAM-1 stimulation, might contribute to cerebral TNF-α production by astrocytes, and thus, participate in an amplification of the inflammatory response. Moreover, TNF-α can affect astrocytes by induction of MHC class I molecule and by overexpression of ICAM-1 and MHC class II, which may lead to a further enhancement of leukocyte adhesion and activation. Interestingly, recent observations indicate that following TNF-α-induced overexpression, ICAM-1 is shedded from astrocytes as a soluble form, which may block its receptor on immune effector cells, and thus, limit the inflammatory response by a negative feedback mechanism (52). In addition, TNF-α has been implicated in blood-brain barrier disruption in patients with multiple sclerosis (53) and bacterial meningitis (24). This suggests that ICAM-1 binding on astrocytes may be a stimulus for blood-brain barrier opening. Together with our recent report that ICAM-1 cross-linking on brain endothelial cells leads to cytoskeleton modification and may directly participate in tight junction opening (12), our present results indicate that ICAM-1 stimulation on astrocytes may play a significant role in the development of inflammatory processes within the CNS and may explain its contribution to the pathogenesis of neuroinflammatory diseases.
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