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Regulation of astrocyte proliferation by prostaglandin E_2 and the α subtype of protein kinase C

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We found that astrocytes expressed the α subtype of protein kinase C. Treatment with 12-*O*-tetradecanoylphorbol 13-acetate (TPA) caused cultured astrocytes to proliferate. This effect of TPA was blocked by staurosporine, a potent protein kinase C inhibitor, suggesting the involvement of protein kinase C in astrocyte proliferation. Indomethacin, an inhibitor of prostaglandin formation, enhanced both the normal and TPA-induced proliferation of astrocytes. Authentic prostaglandin E_2 blocked this effect of indomethacin and also partially blocked the effect of TPA, suggesting that the intracellular mechanisms involved in prostaglandin E_2-regulated astrocyte growth might differ from those acting in protein kinase-dependent growth. The effect of prostaglandin E_2 was blocked by a specific anti-prostaglandin E_2 polyclonal antibody. Cultured astrocytes and microglia produced and released prostaglandin E_2 in response to stimulants such as lipopolysaccharide, TPA, and lymphokines. Since the sensitivity of astrocytes and microglia to these stimuli was different, prostaglandin E_2 may differentially regulate astrocyte proliferation under different physiological conditions, acting in an autocrine fashion for astrocytes and in a paracrine fashion for microglia.

INTRODUCTION

Astrocytes are a type of glial cell and they provide structural support for neurons. There is also growing evidence that astrocytes have additional functions since they have been shown to synthesize and/or respond to a variety of growth factors[20] and cytokines, including interleukin 1[11], interleukin 6[7,43], granulocyte-macrophage colony stimulating factor[19,31], and tumor necrosis factor α[40,43]. It has also been demonstrated that certain factors control both the activity and proliferation of astrocytes via intracellular signaling mechanisms. Protein kinase C is one of the key enzymes involved in intracellular signaling and its activation has been implicated in a wide range of cellular processes[27,28]. One of the most important roles of protein kinase C is modulation of the process of cell growth and division[1,27]. Brain tissue has the highest protein kinase C content in the body[15,23] and the density and distribution of phorbol ester binding sites in the fetal brain suggest a role for this enzyme in both developmental processes and cell growth[25]. The existence of protein kinase C has also been demonstrated in primary astrocyte cultures[37] and it has been shown that mediators such as neurotransmitters or growth factors can stimulate protein kinase C activity[26]. A phorbol ester was recently shown to induce the proliferation of cultured astrocytes[2]. Therefore, protein kinase C seems to be involved in the promotion of astrocyte growth both in the developmental stage and under certain pathological conditions.

Molecular cloning of the cDNA for protein kinase C has recently clarified the existence of multiple subtypes of this enzyme[5,16,18,30,32,36], and there appear to be at least seven subtypes (α–ζ, including two β subspecies)[29]. The mammalian brain has been shown to contain at least four subtypes, α, βI, βII, and γ[33], by immunohistochemical analysis; they were localized in neurons[38] and in glial cells[10].

Like the cells of the immune system, brain cells such

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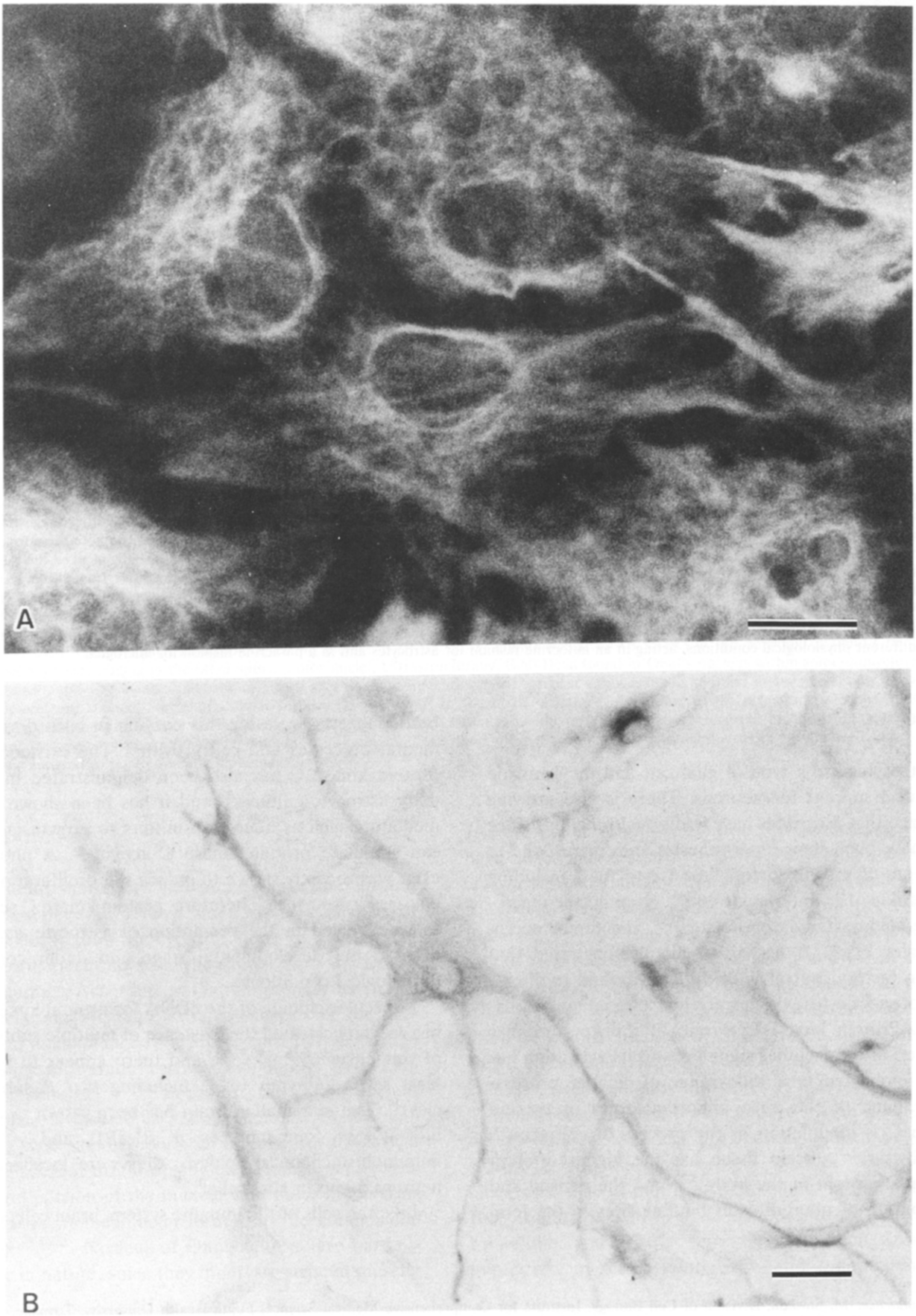

Fig. 1. A: staining of cultured astrocytes for GFAP. B: staining of cultured astrocytes for the α subtype of protein kinase C. Bars in A and B = 15 and 30 μm, respectively.

as astrocytes[8] and microglia[47] have also been shown to produce prostaglandin E_2, although its function in the central nervous system is as yet unknown. An attractive hypothesis is that growth factors or cytokines may activate astrocyte proliferation and subsequently induce prostaglandin E_2 production, which then down-regulates astrocyte growth in an autoregulatory circuit. This hypothesis has been supported by a number of studies on lymphocytes, which have demonstrated that prostaglandin E_2 suppresses the activity or growth of both macrophages and T cells[9,12,13]. However, the effects of prostaglandin E_2 on astrocyte proliferation have not yet been investigated directly.

In this study, we showed that a phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate (TPA) could induce astrocyte proliferation, most likely via activation of the α subtype of protein kinase C, and that prostaglandin E_2 inhibited both the normal and TPA-stimulated proliferation of cultured astrocytes.

MATERIALS AND METHODS

Materials

Bovine insulin, indomethacin, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT), and lipopolysaccharide (LPS) were obtained from Sigma (St. Louis, MO, USA). Prostaglandin E_2 and anti-prostaglandin E_2 antibody were obtained from Funakoshi (Tokyo, Japan). Staurosporine was purchased from Kyowa Medex (Tokyo, Japan). All other reagents used were of the highest purity commercially available. A crude lymphokine preparation was obtained by the method of Suzumura et al.[46].

Preparation of astrocytes and microglia

Astrocytes were prepared from primary mixed glial cell cultures of normal newborn ICR mouse and were purified by 3–4 cycles of trypsinization and replating, as described previously[40]. The purity of the astrocytes thus obtained was more than 95% as determined by indirect immunofluorescence using an anti-glial fibrillary acidic protein antibody[46]. These astrocyte-enriched cultures did not contain neurofilament-positive neurons[44] and the contamination of microglia was negligible[45]. Microglia were prepared as described previously[40,42]. The purity of microglia was more than 98% as determined by indirect immunofluorescence using an anti-MacI antibody.

Immunostaining of protein kinase C subtypes

Purified astrocytes were plated on 14 mm diameter glass coverslips at a density of 2.5×10^4 cells/ml. After 3 days of culture, they were immunolabeled with isozyme-specific anti-protein kinase C antibodies (directed against the α, β and γ subtypes), using a commercially available protein kinase C staining kit (MBL, Nagoya, Japan).

Treatment of astrocytes

Isolated astrocytes were seeded at a density of 2.5×10^4 cells per well in 24-well Falcon testplates. After 12 h of culture, they were stimulated with TPA, LPS, or a crude lymphokine preparation. For the inhibition test, a potent protein kinase inhibitor, staurosporine[48], was added to the medium in the presence of 100 ng/ml TPA. In addition, various concentrations of indomethacin (an inhibitor of prostaglandin production) were added to the medium in the presence or absence of 100 ng/ml TPA. Finally, prostaglandin E_2 with or without an anti-prostaglandin E_2 antibody was added to the medium in the presence or absence of TPA or indomethacin. Cultures were maintained in Eagle's MEM supplemented with 3.5 g/l glucose and 5 mg/l bovine insulin for 3 or 5 days at 37°C, and then assayed.

Measurement of cell proliferation

The proliferative activity of astrocytes was determined by a modification of the MTT colorimetric method[24]. MTT is cleaved by living cells to yield a formazan product and this process requires active mitochondria. Thus, measurement of the formazan product by a colorimetric assay gives an indication of the number of surviving cells[42]. Cells in 24-well test plates were washed twice with phosphate-buffered saline (pH 7.2) and treated with 0.5 ml of a culture medium containing 0.5 mM MTT. After incubation at 37°C for 6 h, 0.04 M HCl-isopropanol was added, the amount of the formazan product in 0.2 ml of the culture fluid was measured at OD 620 nm with a J2000 Immuno Reader (Inter Med Japan Co., Tokyo, Japan).

Measurement of prostaglandin E_2

Prostaglandin E_2 concentrations in the medium from cultured astrocytes and microglia were measured by radioimmunoassay with an anti-prostaglandin E_2 antibody (Amersham Japan, Tokyo, Japan).

Statistics

Control and experimental values were compared using Student's *t*-test.

RESULTS

The purity of the cultured astrocytes was determined to be more than 95% by GFAP immunostaining with the anti-GFAP antibody (Fig. 1A). These astrocytes contained the α subtype of protein kinase C (Fig. 1B), but β and γ subtypes were not detected in our experiment (data not shown).

The amount of formazan product detected at 620 nm showed a good correlation to astrocyte numbers (Fig. 2), so the colorimetric assay was validated for assessing astrocyte proliferation in the subsequent experiments.

When astrocyte cultures were treated with three stimulants (TPA, LPS and crude cytokine extract) that have been shown to activate these cells in different manners[39], only TPA was found to enhance astrocyte proliferation (Fig. 3A). TPA enhanced astrocyte proliferation in a dose-dependent manner from a concentration of 0.1 to 100 ng/ml, but was toxic at higher concentrations (Fig. 3B). Since the cultures were not confluent, the astrocytes gradually increased in numbers, showing a 25% increase on day 3 and a 45% increase on day 5 (Fig. 3C). This time-dependent increase was enhanced by TPA to about 40% on day 3 and 55% on day 5, when compared to the respective control (Fig. 3C). The effect of TPA was inbibited by 10 nM staurosporine (Fig. 3C).

Indomethacin (1 μM) increased both the control and TPA-induced proliferation of astrocytes by 260% and 370%, respectively (Fig. 4). This effect of indomethacin showed saturation at around 10 μM. It

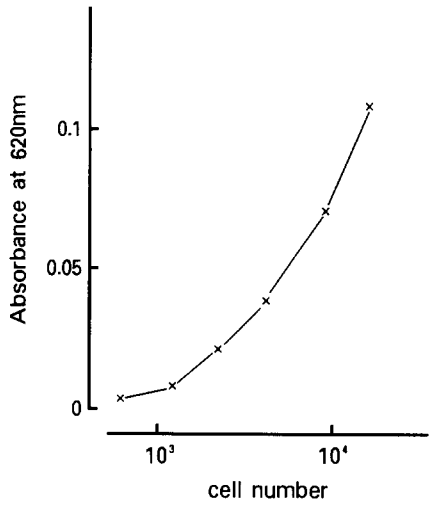

Fig. 2. The relationship between astrocyte numbers and formazan production from MTT. The indicated number of astrocytes were seeded in a 24-well testplate, cultured for 5 days and subjected to the MTT assay as described in Materials and Methods. The amount of formazan produced was measured at 620 nm. Each value indicates the mean of quadruplicate samples from two different experiments.

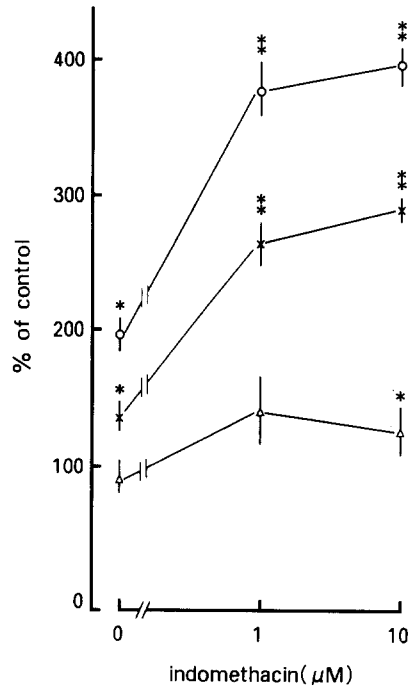

Fig. 4. Effects of indomethacin on astrocyte proliferation. Astrocytes (2.5×10^4) were incubated for 5 days with the indicated concentrations of indomethacin and (○) 100 ng/ml TPA (△) 100 ng/ml TPA + 10 nM staurosporine, or (×) medium only. Points and bars represent the mean ± S.D. of 5 different experiments and show the percentage of the value on day of treatment. The control value is 0.098 ± 0.011. * $P < 0.05$. ** $P < 0.01$.

also increased astrocyte proliferation in the presence of 10 nM staurosporine, which completely inhibited TPA-dependent growth; 10 μM indomethacin increased astrocyte proliferation slightly but significantly (Fig. 4).

Authentic prostaglandin E_2 inhibited both the indomethacin-induced and control proliferation of astrocytes (Fig. 5), and it reduced the TPA-induced proliferative response of astrocytes to only 30% (Fig. 5). These effects of prostaglandin E_2 were reversed by addition of the anti-prostaglandin E_2 antibody (Fig. 5). Treatment with this antibody increased astrocyte prolifera-

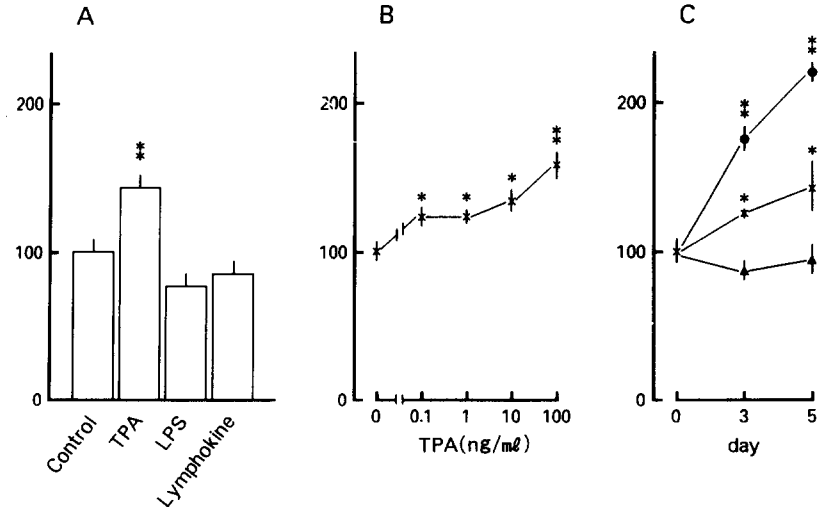

Fig. 3. A: effects of various stimulants on astrocyte proliferation. Astrocytes (2.5×10^4) were incubated with 100 ng/ml of TPA, 1 μg/ml LPS, 10% (v/v) crude cytokine extract, or medium alone (control) for 5 days. Columns and bars represent the mean ± S.D. from 5 different experiments and shown the percentage difference from untreated control cultures (which were taken as 100%). The control value was 0.122 ± 0.012. ** $P < 0.01$. B: dose-dependent increase of astrocyte proliferation in response to TPA. Astrocytes (2.5×10^4) were incubated with the indicated concentrations of TPA for 5 days. Points and bars represent the mean ± S.D. of 3 different experiments and show the percentage of the untreated control proliferation. The control value was 0.135 ± 0.010. * $P < 0.05$. ** $P < 0.01$. C: time course of astrocyte proliferation. Astrocytes (2.5×10^4) were incubated with 100 ng/ml TPA in the presence (▲) or absence (●) of 10 nM staurosporine or medium alone (×) for the indicated number of days. Points and bars represent the mean ± S.D. of 5 different experiments and show the percentage of the value on day 0 of culture. The control value was 0.098 ± 0.011. * $P < 0.05$. ** $P < 0.01$.

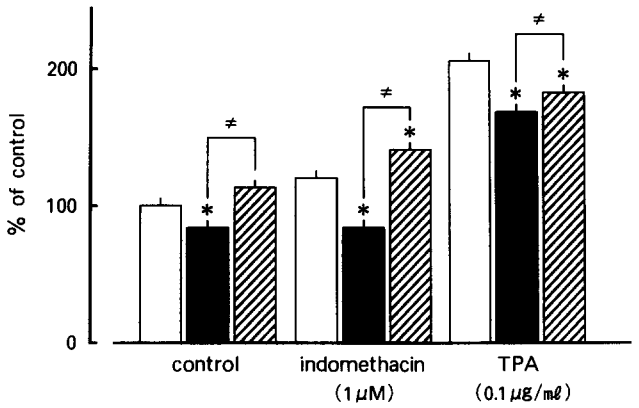

Fig. 5. Effects of prostaglandin E_2 on astrocyte proliferation. Astrocytes (2.5×10^4) were incubated with prostaglandin E_2 (5 mg/ml was added once daily for 5 days) in the presence (hatched columns) or absence (filled columns) of an anti-prostaglandin E_2 antibody (1:500 dilution) or medium only (open columns). Similar experiments were performed in the presence of 1 μM indomethacin or 100 ng/ml TPA. Columns and bars represent the mean ± S.D. of 6 different experiments and show the percentage of the untreated control value. The control value was 0.125 + 0.008. * $P < 0.05$ when compared to the respective control. ≠ $P < 0.05$ when compared as indicated.

tion in the presence or absence of indomethacin to 112% and 140% of the respective prostaglandin E_2-treated levels.

Astrocytes produced about 200 pg of prostaglandin E_2 per 2×10^5 cells under control culture conditions, an amount that was four times the production by microglia under the same conditions (Fig. 6). LPS and TPA increased prostaglandin E_2 production about 1.7-fold and 4.5-fold, respectively, when compared to the control astrocyte cultures. LPS and TPA also increased microglial prostaglandin E_2 production by

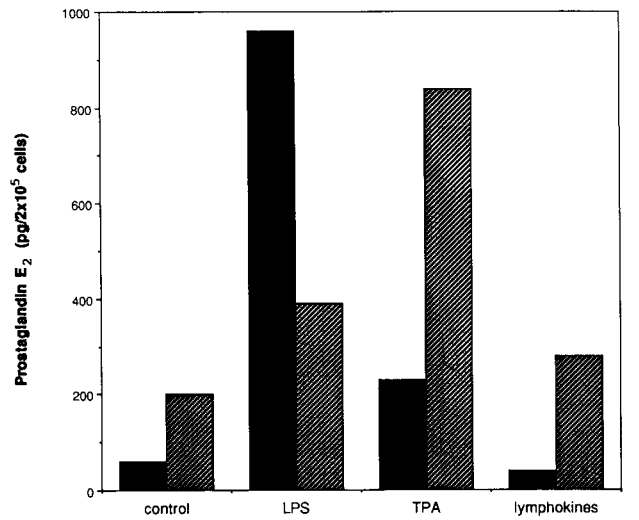

Fig. 6. Production of prostaglandin E_2 by astrocytes and microglia. Astrocytes (5×10^5, hatched columns) and microglia (5×10^5, filled columns) were incubated with or without LPS (0.1 μg/ml), TPA (0.1 μg/ml), or crude lymphokines (10%) for 24 h. Supernatant fractions were then collected and the prostaglandin E_2 content was measured with a radioimmunoassay kit. The values indicate the mean of two independent experiments.

about 40-fold and 5-fold, respectively. Thus, astrocytes were more sensitive to TPA than to LPS, while microglia were the opposite.

DISCUSSION

There are two major aspects to consider with regard to astrocyte proliferation, one being its contribution to normal brain ontogeny[17] and the other being its pathological role in causing gliosis in the mature brain[17,22]. In the latter situation, quiescent astrocytes re-enter the cell cycle like somatic cells such as skin fibroblasts, gut epithelial cells, and hepatocytes[34,35]. Despite the physiological and pathological importance of this cell, relatively little is known about the intracellular and intercellular mechanisms regulating astrocyte proliferation.

We demonstrate here that TPA stimulated the proliferation of cultured astrocytes, and that this effect was blocked by staurosporine, a potent protein kinase C inhibitor[48] (Fig. 2). Staurosporine also inhibited unstimulated astrocyte proliferation (Fig. 2). These observations suggest that protein kinase C may be involved in astrocyte proliferation under both unstimulated and TPA-stimulated conditions. This hypothesis was supported by the result that astrocytes expressed protein kinase C (Fig. 1B). Glial cells are reported to be rich in immunoreactive protein kinase C[10] and a [^{3}H]phorbol ester has been found to bind to glial cells at a high level[4]. In general, when cells are stimulated with TPA, protein kinase C activity is translocated from the cytosol to the membrane[2] or to the nucleus[27]. Translocation of its activity to the nucleus seems to be necessary for DNA synthesis, suggesting that protein kinase C may be a key enzyme involved in cell proliferation. A similar mechanism may be involved in the astrocyte proliferation.

Indomethacin, a prostaglandin synthesis inhibitor, enhanced both unstimulated and TPA-stimulated astrocyte proliferation (Fig. 4), suggesting that astrocyte-derived prostaglandins reduced its own proliferation in both unstimulated and TPA-stimulated conditions. To identify what type of prostaglandins inhibit astrocyte proliferation, we added the authentic prostaglandins to astrocyte cultures. An excess of prostaglandin E_2 reduced both unstimulated and stimulated proliferation of astrocytes (Fig. 5). This inhibitory effect was blocked by an antibody to prostaglandin E_2 (Fig. 5). These findings indicate that this prostaglandin apparently functions as an inhibitory regulator of astrocyte proliferation.

We also demonstrated that prostaglandin E_2 partially blocked the effect of TPA on astrocytes (Fig. 5), suggesting that different intracellular signaling mecha-

nisms are operative for these two agents. This hypothesis was supported by the following two lines of evidence: (1) the effects of indomethacin and TPA were additive, even in the presence of such a high concentration of indomethacin that its effect was almost saturated (Fig. 4), and (2) indomethacin increased the proliferation of astrocytes even in the presence of staurosporine (Fig. 4). The details of the relationship between the TPA-protein kinase C system and prostaglandin E_2 are not yet clear, however, with respect to the effect on astrocyte growth.

Astrocytes and microglia produced prostaglandin E_2 in response to several stimuli (Fig. 6), a finding which was consistent with previous reports[8,14]. The stimuli used in this experiment were designed to represent different physiological and pathological conditions[41]. Thus, the addition of LPS, TPA, and the crude lymphokine preparation, respectively, represented the effect of bacterial antigens on the brain, the effect of protein kinase C activation by growth factors[42] and the effect of peripheral immune activation on the brain. Prostaglandin production by astrocytes and microglia differed in response to these stimuli (Fig. 6), suggesting that this prostaglandin may regulate astrocyte proliferation in a differential manner. That is, in astrocytes it may act in an autocrine fashion and be sensitive to phorbol ester stimulation, while in microglia it appears to act in a paracrine fashion and be sensitive to LPS stimulation.

Prostaglandin E has inflammatory and immunomodulatory properties. Since astrocytes and microglia have been suggested to act as immunoregulatory cells in the central nervous system[6,49], the release of this prostaglandin from these cells may be a key element in the central nervous system immune response. An increased central nervous system level of prostaglandin E_2 has been reported in multiple sclerosis[21] and in experimental allergic encephalomyelitis[3]. The production of prostaglandin E_2 by astrocytes in such conditions may have some role in their pathogenesis, since our study showed that this prostaglandin was not only involved in immunomodulation but also in regulating astrocyte growth.

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ABBREVIATIONS

GFAP glial fibrillary acidic protein
LPS lipopolysaccharide
MTT 3-[4,5-demethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
TPA 12-*O*-tetradecanolyphorbol 13-acetate

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