Immunocontent and secretion of S100B in astrocyte cultures from different brain regions in relation to morphology

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Received 18 October 2000; revised 14 November 2000; accepted 15 November 2000

First published online 29 November 2000

Edited by Guido Tettamanti

Abstract Primary astrocyte cultures prepared from neonatal hippocampus, cerebral cortex and cerebellum were morphologically distinct. Cells from hippocampus and cortex were almost entirely protoplasmic, whereas cerebellar astrocytes had many processes; in the absence of serum these differences were accentuated. We compared the immunocontent and secretion of the mitogenic protein S100B in these cultures. Immunocontent was 2.5 times higher in cerebellar astrocytes than in hippocampal or cortical astrocytes. Cells from all three regions secreted S100B under basal conditions, but the secretion rate was higher in cerebellar astrocytes. Secretion depended on protein synthesis and was increased by incubation with forskolin or lysophosphatidic acid in mechanisms which were additive. The stellate morphology induced by forskolin was reversed by lysophosphatidic acid in hippocampal but not in cerebellar cultures, suggesting that S100B secretion was not associated with a process-bearing phenotype of astrocytes.

Key words: S100B; Astrocyte culture; Brain region; Glial fibrillary acidic protein; Morphology

1. Introduction

It is now generally recognized that astrocytes are heterogeneous in morphology and biochemistry with diverse functions and complex interactions with neurons [1–5]. Astrocytes synthesize various growth factors, cytokines and components of the extracellular matrix, some of which have been shown to rescue damaged neurons in model lesions of the CNS [6–8]. Biochemically distinct astrocytes have been demonstrated in primary cultures of astrocytes [9] and such astroglial heterogeneity in situ may reflect regional specializations in local neurons. Moreover astrocytes from different brain regions and at different developmental stages are heterogeneous in multiple features including receptors and ion channels [10,11].

The protein S100B belongs to the S100 family of calcium binding proteins and is considered a marker of astrocytes [12]. The protein is secreted by astrocytes and plays a neurotrophic role in neighboring cells, as well as possessing autocrine functions regulating astrocytic proliferation [13]. Further many studies have suggested an intracellular role for S100B particularly in the regulation of the cytoskeleton. Considering the importance of the cytoskeleton in regulating cell morphology it appeared possible that variation in the synthesis, content and secretion of S100B might be associated with morphological variation. To investigate this possibility we have studied astrocyte morphology and the immunocontent and secretion of S100B in primary cultures prepared from three different brain regions.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), flasks and other material for cell culture, acrylamide, forskolin, lysophosphatidic acid (LPA) and cycloheximide and antibodies against glial fibrillary acidic protein (GFAP) (polyclonal, G9269) and S100B (clone SHB1) were purchased from Sigma. Fetal calf serum was purchased from Gibco; horseradish peroxidase-conjugated secondary antibodies and nitrocellulose membranes were from Amersham; peroxidase-conjugated polyclonal anti-S100 came from Dako. For GFAP immunocytochemistry a polyclonal antibody (20334) from Dako was used.

2.2. Astrocyte cultures

Primary astrocyte cultures were prepared as previously described [14]. Briefly, hippocampus, cortex and cerebellum of newborn Wistar rats (1–2 days old) were removed, placed in Ca^{2+} and Mg^{2+}-free balanced salt solution (CMF-BSS; pH 7.4, containing 137 mM NaCl; 5.36 mM KCl; 0.27 mM Na_2HPO_4; 1.1 mM KH_2PO_4 and 6.1 mM glucose), cleaned of meninges and mechanically dissociated by sequential passage through a Pasteur pipet. The cell suspension was centrifuged at 1000 rpm for 5 min. The supernatant was discarded, the pellet resuspended in culture medium (pH 7.6) containing 1% DMEM; 8.39 mM HEPES; 23.8 mM $NaHCO_3$; 0.1% fungizone; 0.032% garamicine and 10% fetal calf serum. The cells were plated at a density of 1.5×10^5 cells/cm^2 onto 24 well plates pre-treated with poly-L-lysine. Cultures were maintained in 5% CO_2/95% air at 37°C and allowed to grow to confluence (20–24 DIV).

2.3. Gel electrophoresis and immunoblotting

Stopping solution (110 μl, 2.1 mM EDTA, 50 mM Tris–HCl, 4% SDS, pH 6.8) was added and the cells were scraped and transferred to Eppendorf tubes. Samples were boiled for 2 min, cooled and 25 μl of a solution containing 40% glycerol, 5% mercaptoethanol, 50 mM Tris–HCl, pH 6.8 and a trace of bromophenol blue was added. The samples were applied to 10% gels. Following electrophoresis the proteins were transferred to nitrocellulose and immunoblotting for GFAP carried out as described previously [14]. Briefly, the membranes were blocked with 5% defatted milk in TBS medium (20 mM Tris, 500 mM

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Abbreviations: GFAP, glial fibrillary acidic protein; LPA, lysophosphatidic acid

0014-5793/00/$20.00
PII: S0014-5793(00)02301-2

NaCl, pH 7.5) for at least 2 h. Then the membranes were incubated with 1:500 anti-GFAP in milk-TBS for 1 h and finally with 1:3000 peroxidase-conjugated secondary antibody also for 1 h. Conjugates were developed by the ECL method, using a kit from Amersham and X-Omat film from Kodak.

2.4. Quantification and analysis of GFAP

This was done by scanning the ECL films in a Hewlett-Packard ScanJet 6100C and determining optical densities with Optiquant (version 02.00, Packard instrument) software. The results were expressed as a percentage normalized to the value for the hippocampus.

2.5. Assay of S100B protein by ELISA

Assay of S100B was carried out by ELISA as described by Green et al. [15]. To measure the liberation of S100B from the cultures (subsequently referred to as 'secretion' since no parallel liberation of LDH was observed) the DMEM medium was replaced with medium without serum and samples were collected at different times during 24 h to analyze the amount of S100B secreted. 50 μl of sample plus 50 μl of barbital buffer (pH 8.6) were applied on microtiter plates previously coated with monoclonal anti-S100B in carbonate buffer and blocked with 1% bovine serum albumin. After washing, peroxidase-conjugated anti-S100 diluted 1:1000 was added and incubation continued for 1 h. The plate was washed and 0.2 ml of peroxidase substrate (Sigma Fast OPD) was added and the plate incubated for a further 30 min in the dark. The absorbance was measured at 492 nm on a microtiter plate reader. The standard curve for S100B was linear between 0.1 and 4 ng/ml. To measure the intracellular content of S100B the medium was removed after 24 h and the cells were dissolved in lysis solution (PBS containing 1 mM PMSF and 1 mM EGTA) and analyzed by ELISA. After 24 h the cells were scraped and total protein was measured.

2.6. Immunocytochemistry

Confluent cells were fixed with 4% paraformaldehyde in PBS for 30 min and then treated with 0.2% Triton X-100 in PBS for 10 min and blocked with 5% BSA for 1 h. Cells were incubated overnight with 1:200 diluted polyclonal anti-GFAP or monoclonal anti-S100B at room temperature. Then the cells were incubated with second anti-

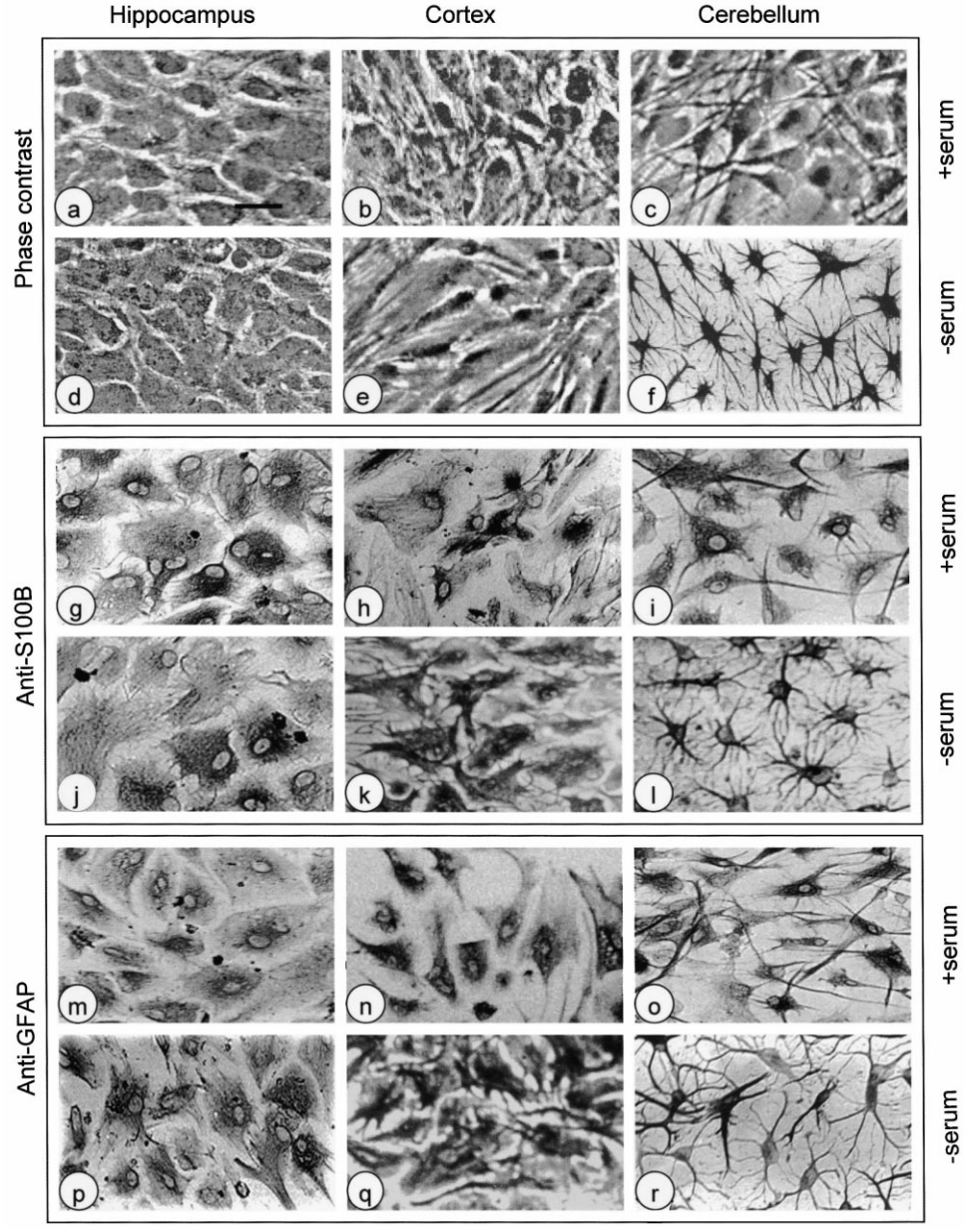

Fig. 1. Morphology of astrocytes in primary cultures prepared from different brain regions in the presence or absence of serum. Cell cultures were prepared from neonatal rat brain as described in Section 2 and allowed to grow to confluence (20–24 DIV) before viewing by phase-contrast optics (a–f) and fixing for immunocytochemistry for S100B (g–i) and GFAP (m–r). Scale bar: 50 μm.

body diluted 1:200 (peroxidase-conjugated anti-rabbit) for 2 h. Finally, cells were incubated for 10 min with 0.05% DAB/0.3% hydrogen peroxide.

2.7. Image analysis

Cells were viewed in a Nikon Eclipse TE300 inverted microscope. Before photographing images typical of a particular parameter at least eight fields were examined.

3. Results

3.1. Morphological differences in astrocyte cultures

Astrocyte cultures prepared from hippocampus, cerebral cortex and cerebellum and grown for 20–24 DIV in the presence of serum, showed clear morphological differences in phase-contrast microscopy (Fig. 1a–c). There were many processes in astrocytes from cerebellum, few in astrocytes from cortex and hardly any in astrocytes from hippocampus. In absence of serum for 2 h these differences were still clearer because of a dramatic increase in processes in cerebellar astrocytes in contrast to almost unchanged astrocytes from hippocampus and only partial stellation in cortical astrocytes (Fig. 1d–f). Interestingly, process formation in cortical astrocytes induced by forskolin (and to a lesser extent by serum deprivation) was different from the other structures: cell bodies were elongated and processes were generally parallel rather than stellate (Figs. 1e and 5Be). Immunocytochemistry for GFAP and S100B, two marker proteins for astrocytes, confirmed these observations, as well as showing the purity of the cultures where more than 98% cells were GFAP- and S100B-positive (Fig. 1g–r), thus excluding the possibility that the morphological differences may be due to contaminating cells. Note the predominantly perinuclear location of the immunoreactivity to S100B, evident particularly in the hippocampus and previously reported in C6 glioma cells [16].

The cerebellum in situ contains a characteristic form of radial glia known as the Bergmann glia, which are related to astrocytes as they express GFAP. Whether Bergmann glia were present in our cerebellar cultures is not known as a characteristic marker for these cells is not available. However the morphological homogeneity of the stellar cerebellar astrocytes in our cultures (Fig. 1f,i,r) suggested that if Bergmann glia were growing under our conditions they were developing into a radically different phenotype from the typical morphology they exhibit in situ.

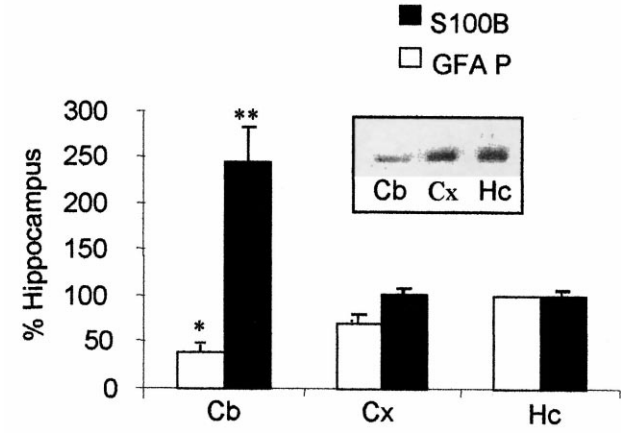

Fig. 2. Immunocontent of GFAP and S100B in astrocyte cultures prepared from cerebellum (Cb), cerebral cortex (Cx) and hippocampus (Hc). GFAP was measured by immunoblotting and densitometric scanning of the ECL films (see inset) and S100B by ELISA as described in Section 2. Equal amounts of protein were analyzed. Results are the mean (±S.E.M.) of 3–6 observations and are expressed as percentages of normalized values for hippocampus. Significantly different from hippocampus $^{**}P<0.001$; $^{*}P<0.01$ (ANOVA).

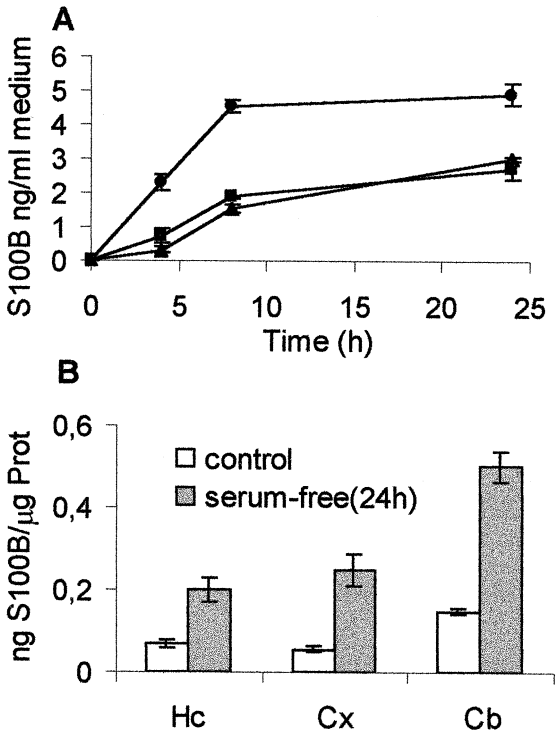

Fig. 3. Time-dependent release (A) and cell content (B) of S100B from astrocyte cultures incubated in the absence of serum. (A) The DMEM culture medium was replaced with medium without serum and aliquots (50 μl) were collected at different times to analyze the amount of secreted S100B. (B) Intracellular content of S100B was measured in control and serum-free incubated cells for 24 h. Points are the mean (±S.E.M.) of 3–6 observations. Cb, cerebellum; Cx, cerebral cortex; Hc, hippocampus.

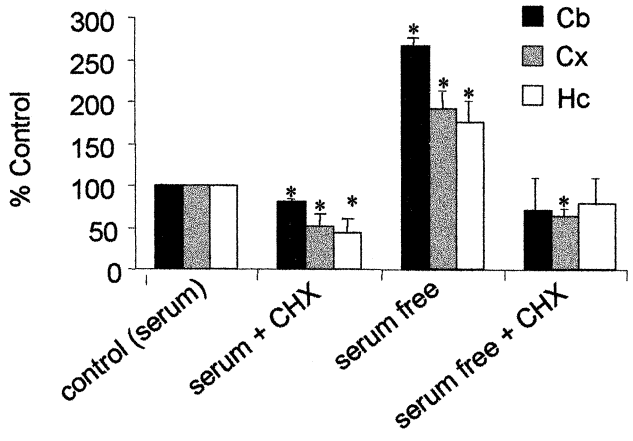

Fig. 4. Inhibition of S100B secretion by cycloheximide. Secretion of S100B was determined as indicated in Fig. 3 after incubation for 24 h in the presence or absence of serum and of cycloheximide (0.5 μg/ml). Results of four observations are expressed as percentages of the normalized values for cells incubated with serum. *Significantly different from control ($P<0.01$).

3.2. Immunocontent and secretion of S100B in astrocytes from different regions

In addition to the morphological differences we found significant differences in the immunocontent of GFAP and S100B in astrocytes prepared from the three regions. The S100B immunocontent of cerebellar astrocytes was nearly 2.5 fold higher than the level observed in hippocampal astrocytes, whereas the cerebellar GFAP immunocontent was approximately one half of the hippocampal content (Fig. 2). Cortical contents of these proteins were not significantly different from hippocampal levels. Astrocytes from all three regions secreted S100B when incubated in the absence of serum for 24 h, but the secretion rate and the total amount secreted from cerebellar astrocytes was strikingly higher than from cortical or hippocampal astrocytes (Fig. 3A). Secretion was accompanied by significant increases in the immunocontent of S100B in the cells (Fig. 3B) and was dependent on protein synthesis as it was inhibited by cycloheximide (Fig. 4). In all three astrocyte cultures secretion was markedly greater in the absence of serum.

In an attempt to discover whether the secretion of S100B was associated with differences in morphology we incubated the astrocytes with forskolin and the lipid mitogen LPA. Forskolin, through its ability to increase intracellular cyclic AMP, is well known to induce stellation in astrocyte cultures [17] and LPA, through its action in activating the small GTPase RhoA, causes most stellar astrocytes to revert to the rounded protoplasmic morphology [18,19]. Both compounds significantly increased S100B release in all three cultures when incubated in the absence of serum through mechanisms which were additive (Fig. 5A). However, as shown in Fig. 5B, secretion was not associated with a process-bearing morphology: thus, hippocampal astrocytes were protoplasmic after incubation with LPA and stellate after forskolin, but in both situations the secretion of S100B was approximately equal. Moreover, when forskolin-induced stellation in hippocampal and

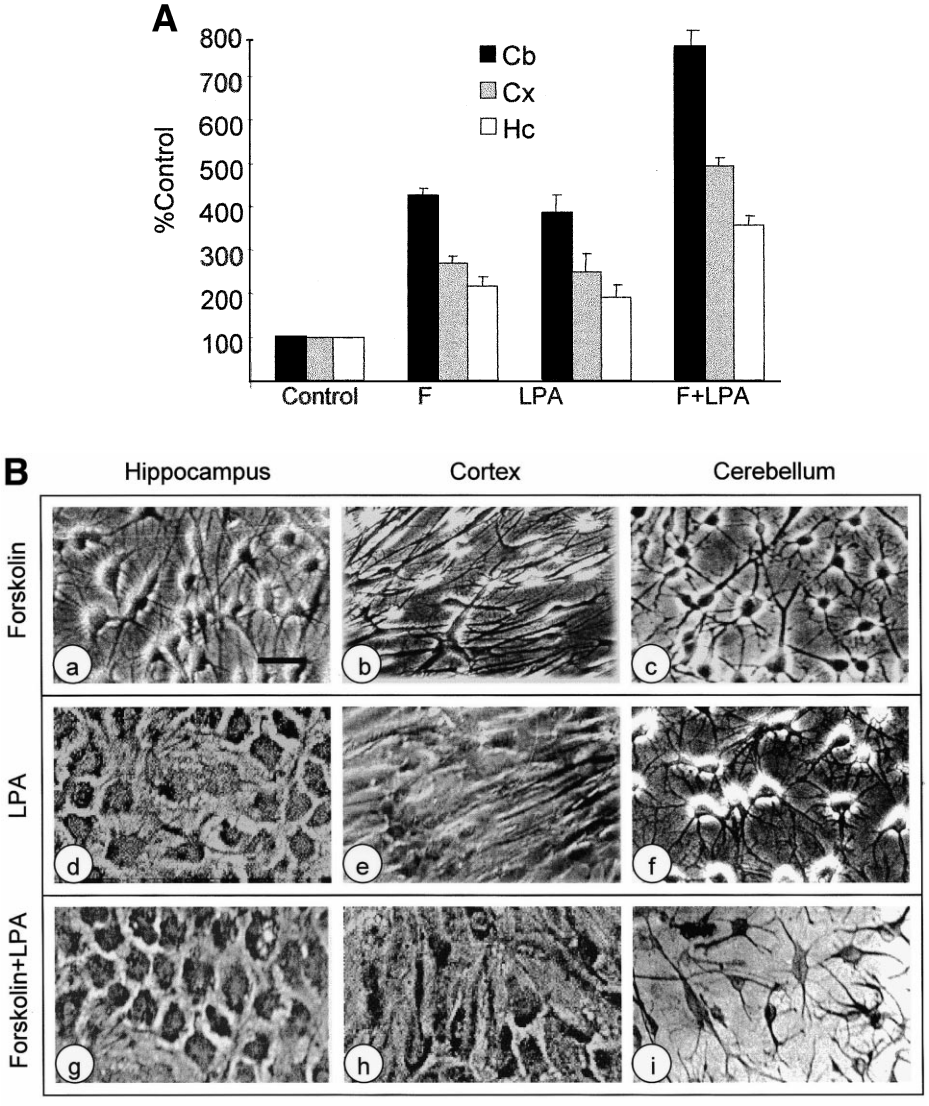

Fig. 5. Effects of forskolin and LPA on the secretion of S100B (A) and on the morphology of cultured astrocytes from three regions (B). Cells were incubated in serum-free medium in the presence of 10 μM forskolin or 2 μM LPA. A: Secretion. The concentration of S100B in the medium was measured by ELISA after incubation for 2 h. Results are expressed as percentages of normalized values for control cells. Equal amounts of protein were analyzed. Each bar represents the mean of 3–6 observations ± S.E.M. All experimental values are significantly different from control ($P < 0.01$). B: Morphology. Phase-contrast images were photographed after incubation for 2 h. Scale bar: 50 μm.

cortical astrocytes was reversed by LPA, secretion of S100B was further increased. By contrast LPA had no effect on the stellation induced in cerebellar astrocytes by serum-deprivation or forskolin, an observation which merits further investigation (compare Fig. 1f with Fig. 5Bf,i).

4. Discussion

4.1. Morphological variation

We found that astrocyte cultures from three brain regions were morphologically distinct and these differences were increased on withdrawal of serum for 2 h, confirming that the phenotypic fate of astrocytes is determined in embryogenesis and neonatal astrocytes are already programmed to develop a characteristic morphology. Cell morphology depends on signal transduction pathways regulating the assembly of cytoskeletal proteins, particularly actin [20,21]. The integrity of the actin cytoskeleton is regulated by the Rho family of small GTPases [22,23] and in astrocytes stellation is associated with down regulation of RhoA and depolymerization of F-actin [19,22]. In hippocampal and cortical astrocytes, stellation is reversed by LPA which up regulates the activity of RhoA [19,23]. It was especially striking therefore to observe that in cerebellar astrocytes stellation induced by serum deprivation or forskolin was not reversed by LPA. This result suggests that some aspects of the control of the actin cytoskeleton by Rho family GTPases in cerebellar astrocytes are different from the other structures.

4.2. Secretion of S100B in astrocyte cultures from three regions

The relatively high rate of secretion of S100B from cerebellar astrocytes was associated with a higher immunocontent compared with hippocampal or cortical astrocytes. The significance of this observation in relation to regional functional variation is unknown. However S100B has extracellular roles [12]. For example S100B stimulates the proliferation of astrocytes and C6 glioma cells [24] and the survival and extension of neurites in neuronal cultures [25]. We have shown that treatment of astrocyte cultures with S100B activates ERK [26], a result which may be related to the effect of the protein on glial proliferation [24].

The increased secretion of S100B following treatment of cells with forskolin (Fig. 5A) was expected since immunodetectable S100B is increased by agents that increase cyclic AMP in astrocytes [27,28]. However, the increased secretion of S100B given by LPA (Fig. 5A) was not observed. This effect of LPA was nearly additive with that of forskolin in all three cultures, pointing to distinct mechanisms. However since the mechanism of S100B secretion from cells is not understood it is not possible to draw any conclusion about the site of action of LPA.

4.3. S100B secretion and immunocontent in relation to morphology

Cytoskeletal targets of S100B include inhibition of the phosphorylation [29] and polymerization [30] of GFAP, modulation of microtubular function [16,31] and the actin-binding protein capZ [32]. Further inhibition of S100B synthesis in cell cultures results in microfilament reorganization and alteration in cellular phenotype [33] and transgenic mice overexpressing S100B show neural cytoskeletal changes [34]. These observations made it of interest to investigate whether S100B secretion was associated with astrocyte morphology, particularly process formation. However we found no evidence for such an association. Presumably this negative result is related to the complexity of the signal transduction pathways that regulate cell morphology.

Acknowledgements: This study was supported by the Brazilian funding agencies CNPq, FINEP (PRONEX).

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