European Journal of Neuroscience, Vol. 11, pp. 2801–2812, 1999

Acidic calponin immunoreactivity in postnatal rat brain and cultures: subcellular localization in growth cones, under the plasma membrane and along actin and glial filaments

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Keywords: astrocytes, development, GFAP, microfilaments, radial glia, vimentin

Abstract

Acidic calponin, an F-actin-binding protein, is particularly enriched in brain, where calponin protein and mRNA are mainly expressed by neurons. The presence of calponin immunoreactivity in cultured astroglial cells has been reported, but the presence of acidic calponin in astrocytes *in vivo* appears equivocal. For the present study, we raised a specific polyclonal antibody against the 16-residue synthetic peptide covering the sequence E311-Q326 (EYPDEYPREYQYGDDQ) situated at the carboxy-terminal end of rat acidic calponin, and we investigated the cellular and subcellular localization of the protein in the developing central nervous system. Our results show that acidic calponin is particularly enriched in: (i) growth cones and submembranous fields of maturing cerebellar and cortical cells, where it codistributes with microfilaments and (ii) glial cells *in vivo*, including radial glia, glia limitans, Bergmann glia and mature astrocytes, and *ex vivo*, where acidic calponin strongly colocalizes with intermediate glial fibrillary acidic protein (GFAP) and vimentin filaments. Finally, up to four acidic calponin subtypes with different isoelectric point (pI) values were identified by two-dimensional gel electrophoresis of cerebellar and hippocampal extracts. The more acidic isoforms were developmentally regulated. As only one single mRNA for acidic calponin has been identified, these isoforms must reflect postsynthesis changes probably related to the particular functions of acidic calponin in maturing cells. Although brain acidic calponin's exact role remains uncertain, the present data suggest that it is involved in neuronal and glial plasticity.

Introduction

Discovered in 1986 and purified from smooth muscle tissues (Takahashi *et al*., 1986), calponin has been characterized as an F-actin, tropomyosin and myosin-binding protein (El-Mezgueldi & Marston, 1996; Vancompernolle *et al*., 1990; Takahashi & Nadal-Ginard, 1991; Mezgueldi *et al*., 1995; Szymanski & Tao, 1997) which inhibits the actin-activated myosin Mg-ATPase activity *in vitro* through a thin filament-associated regulatory system (Abe *et al*., 1990; Winder & Walsh, 1990; Shirinsky *et al*., 1992). For this reason, calponin is thought to play a role in the regulation of smooth muscle cell contraction (as reviewed by Gimona & Small, 1996 and Fattoum, 1997). In addition, calponin induces actin polymerization, stabilizes actin microfilaments (Kake *et al*., 1995) and may be involved in the formation of actin bundles (Kolakowski *et al*., 1995; Tang & Janmey, 1996). More recently, calponin has been shown to interact with smooth muscle desmin intermediate filaments (Wang & Gusev, 1996; Mabuchi *et al*., 1997) and with brain microtubules (Fujii *et al*., 1997), suggesting greater possibilities in the functional role of calponin.

Acidic, neutral and basic isoforms have been identified in various cells and tissues up to date; they are encoded by distinct genes (Takahashi *et al*., 1991; Strasser *et al*., 1993; Applegate *et al*., 1994; Maguchi *et al*., 1995; Masuda et al., 1996). Basic and neutral forms [with an isoelectric point (pI) from 7.8 to 9.9 and molecular weight (Mw) of 34 kDa] are specifically expressed in smooth muscle cells (Takahashi *et al*., 1988; Gimona *et al*., 1990; Strasser *et al*., 1993), though porcine aortic endothelial cell expression has also been reported (Sakihara *et al*., 1996). Acidic forms (with a pI of about 5.2–5.4 and Mw of 36 kDa) seem more ubiquously distributed than the basic ones and are present in both smooth muscle and nonmuscle cells (Applegate *et al*., 1994), and notably in rat and pig brains (Trabelsi-Terzidis *et al*., 1995; Ferhat *et al*., 1996). In contrast to basic calponins, acidic calponin has a unique acidic carboxyl terminus of 57 residues which is particularly enriched in glutamate, aspartate, proline and tyrosine (Applegate *et al*., 1994; Ferhat *et al*., 1996). Like basic forms of calponin, acidic calponin associates with the actin stress fibres in transiently transfected cells (Ferhat *et al*., 1995; Gimona & Mital, 1998) and interacts with F-actin (Applegate *et al*., 1994). Nevertheless, little is known about the function of acidic calponin in nonmuscle cells.

In previous studies, using anti-gizzard calponin antibodies which recognize both acidic and basic isoforms, we demonstrated the presence and the distribution of immunoreactive proteins in the adult rat brain and in rat cerebellar cultured cells (Represa *et al*., 1995; Trabelsi-Terzidis *et al*., 1995). Immunohistochemical analyses support the notion that calponin-immunoreactivity (IR) is restricted to

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Received 7 October 1998, revised 30 March 1999, accepted 7 April 1999

the somatodendritic domains of mature neurons, in which it colocalizes with actin and myosin (Represa *et al.*, 1995). Nevertheless, acidic calponin has also been detected in glial cell cultures where it strongly codistributes in cells stained with the astroglial marker glial fibrillary acidic protein (GFAP), suggesting that acidic calponin is also present in astrocytes (Trabelsi-Terzidis *et al.*, 1995). Furthermore, our group has cloned acidic calponin from the adult rat brain and from hippocampal neurons and astroglial cell cultures (Ferhat *et al.*, 1996). The developmental analysis of mRNA expression by reverse transcriptase polymerase chain reaction and *in situ* hybridization showed that acidic calponin messenger was highly expressed in the immature postnatal rat brain by both Bergmann glial cells and subsets of neurons such as the cerebellar granular cells (Ferhat *et al.*, 1996). However, the localization of the protein in glial cells *in vivo* and in the developing brain is presently unknown. Moreover, we thought that it would be interesting to determine the localization of acidic calponin in the different subcellular compartments of the growing and mature cultured brain cells in order to consider its potential functions in the central nervous system. For this purpose, we raised a specific antibody against the acidic carboxyl terminus of rat brain calponin and, using immunochemical procedures (immunoblots and immunocytochemistry), we compared the spatial and temporal distribution of this protein with neuronal and glial markers. In this study, we describe the expression and the localization of acidic calponin in the developing rat brain cells both *in vivo* and *ex vivo*.

Materials and methods

Purification of proteins

Gizzard calponin was purified according to El-Mezgueldi *et al.* (1996) and rat brain actin according to the method previously described by Prulière *et al.* (1986).

Generation and purification of the antibody to the terminal acidic sequence of the rat brain calponin isoform

The peptide EQ16, representing the 16-residue sequence of the C-terminal region of acid calponin between residues 311 and 326 (EYPDEYPREYQYGDDQ) was prepared by a solid phase method on a continuous flow synthesizer using the Fmoc (9-fluorenylmethoxycarbonyl) aminoacids strategy (Valembois *et al.*, 1992). The peptide was further purified by semipreparative reverse phase HPLC and its expected structure was checked by aminoacid analysis, sequence determination and molecular mass measurement using electrospray ionization mass spectrometry. The concentration of EQ16 was estimated by aminoacid analysis. Polyclonal antibodies directed specifically against the acidic rat calponin (RCp) were raised in rabbit immunized with the synthetic peptide EQ16 coupled to keyhole limpet haemocyanin by the glutaraldehyde method of Bulinski *et al.* (1983). Finally, the antibodies were affinity purified over immobilized EQ16 synthetic peptide on Affigel.

SDS-PAGE and immunoblots

Antibodies used in immunoblots: monoclonal antibody to calponin mCp, at 4 µg/mL (Sigma, clone CP93), polyclonal antibody to purified turkey gizzard calponin GCp92; at 2.5 µg/mL (Mezgueldi *et al.*, 1992), polyclonal antibody specifically raised to the C-terminal acidic sequence of the rat brain calponin isoform RCp at 0.5 µg/mL (see above); and monoclonal antibody to β-actin, at 2 µg/mL (Sigma, clone KJ43 A). All polyclonal antibodies were purified on their respective antigens covalently bound to Affigel (Bio-Rad). The specificity of the antibodies was tested with sodium dodecyl sulphate polyacrilamid gel electrophoresis (SDS-PAGE) and immunoblotting. Electrophoreses and Western blots were performed as previously described (Represa *et al.*, 1995) according to the procedure of Laemmli (1970) and of Towbin *et al.* (1979). Before loading, hippocampus and cerebellum from adult rat (Wistar) perfused with 150 mM NaCl were homogenized in a mixture of 9 M urea and Laemmli's sample buffer (0.062 mM Tris-HCl pH 6.8, 1% β-mercaptoethanol, 7% glycerol and 2% SDS) and boiled for 2 min.

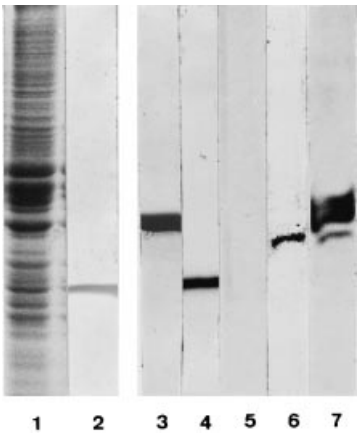


FIG. 1. Electrophoresis and immunoblotting showing the cross-reactivity of the antibodies used in this study. Lane 1, 60 µg of total homogenate of rat hippocampus. Lane 2, 1 µg of purified gizzard calponin. Lanes 1 and 2 are stained with Coomassie brillant blue. Lane 3–7 are immunoblots of: 2 µg of purified rat brain actin stained with antibody to β-actin (Lane 3); 2 µg of purified gizzard calponin stained with the polyclonal antibody to basic calponin GCp92 (Lane 4); the polyclonal antibody raised against the terminal acidic sequence of the rat brain calponin RCp (Lane 5); and 60 µg of hippocampus homogenate labelled with RCp and RCp plus β-actin antibodies (Lanes 6 and 7, respectively).

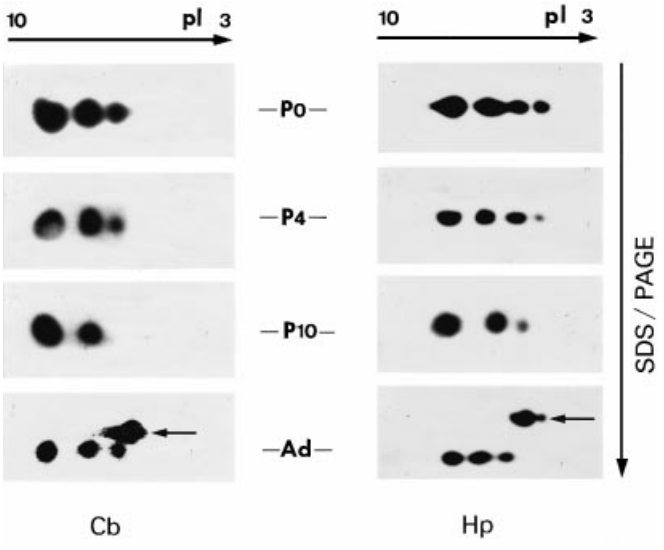


FIG. 2. Immunoblots of two-dimensional gels showing the developmental expression of acidic rat brain calponin isoforms. Several isoforms were detected in Cerebella (Cb) and hippocampi (Hp) homogenates from P0, P4 and P10 rat pups and from the adult (Ad). Note the high amount of calponin at P0 (125 µg total protein) compared with that detected in mature tissues (260 µg total protein). Adult two-dimensional IEF-SDS-PAGE blots were also probed for β-actin in reference to its isoelectric point of 5.5 (small arrows).

Proteins were electrotransferred onto nitrocellulose sheets (0.2 µm, Schleicher & Schuell). Staining was revealed using biotinylated polyclonal antirabbit or monoclonal antimouse (Vector) antibodies used at 1 : 2000 and phosphatase alkalin (Vector) at 1 : 4000. Gels were stained with Coomassie brillant Blue. Protein concentration was determined as outlined by Bradford (1976).

Two-dimensional gel electrophoresis (IEF-SDS-PAGE)

Two-dimensional gels (1 mm × 12 cm) were run essentially according to the method of O'Farrel (1975), using the two-dimensional system of Prolabo. First dimensional gels were prerun successively at 200 V for 15 min, at 300 V for 30 min and at 400 V for 45 min. Whole hippocampus or cerebellum from adult perfused rat were homogenized directly in lysis buffer containing 9.5 M urea and 2% ampholines (Bio-Rad or Pharmacia Biotechnology) covering pH ranges 3–10, 4–6, 5–7, 5–8 and 7–9. Isoelectric focusing was performed at 400 V for 15–18 h. The gel was then rapidly washed with distilled water, incubated for 20 min at room temperature in SDS-Laemmli's sample buffer and submitted to electrophoresis and immunoblotting as indicated above. Hippocampi and cerebella from P0, P4 and P10 rat pups were treated the same way.

Immunohistochemistry and confocal microscopy observation of double-labelled slices

Adult male Wistar rats (180–220 g) and pups (P0, P4, P7, P10, P14 and P21) were anaesthetized with pentobarbital. These animals were killed by perfusion through the heart with physiological saline (0.9% NaCl) to remove the blood and with 0.1 M phosphate buffer (PB) containing 4% paraformaldehyde (PFA). Brains were removed and kept in the same fixative for at least 18 h, and then stored at 4 °C in PB containing 0.2% sodium azide. Coronal, horizontal and sagittal sections (40 µm) of the rat brain were cut with a vibratome. The slices were incubated free-floating, overnight at room temperature, with either polyclonal or monoclonal antibodies diluted in 0.1 M PB/0.5% Gelatin/0.2% or 0.4% Triton X-100 with $MgCl_2$ (3 mM)/EGTA (1 mM). The antibodies were the following: polyclonal antiacidic calponin RCp (optimum concentration at 0.75 µg/mL); polyclonal antibasic calponin GCp92 (optimum concentration at 2.5 µg/mL); monoclonal antivimentin (DAKO; 1 : 200); monoclonal anti-GFAP (DAKO; 1 : 200); monoclonal antiβ III tubulin (Sigma; 1 : 400); monoclonal anti-MAP-2 (microtubule-associated protein 2, Sigma; 1 : 400); and monoclonal antiparvalbumin (Sigma; 1 : 400). After washing, immunoreactivity was revealed with biotinylated antirabbit or antimouse antibodies (from DAKO, 1 : 200) and then amplified with the Vectastain ABC system (from Vector). Horseradish peroxidase activity was revealed with 0.05% diaminobenzidine and 0.01% H_2O_2 in 0.05 M Tris-HCl (pH 7.4). Immunohistochemical controls were performed by omitting either primary or secondary antibodies.

Immunofluorescence double labelling was performed in similar conditions with free-floating slices and appropriate combinations of primary antibodies for at least 18 h of incubation. Immunoreactivities were revealed using secondary antibodies conjugated with either rhodamine or fluorescein (Vector, 1 : 200). The double-labelled preparations were analysed with fluorescence microscopes (Nikon and Olympus) and with a confocal scanning laser microscope (Bio-Rad MRC 600). Confocal optical sections of 5–15 µm, corresponding to the depth of the scanned images, were taken according to the fluorescence signal intensity. Both fluorochromes were simultaneously excited with a krypton/argon ion LASER (488 and 568 nm) and the fluorescent light was filtered (520 and 585 nm). Collected images were projected to show both signals simultaneously and each label was associated with 'pseudocolours'. In the condition of analysis, no cross-over signals were detected. Photographs representative of the staining were taken on the screen.

Cell cultures and immunocytochemistry

For cerebellar granule cell cultures, cerebella were removed from 2 or 4-day-old rat pups under sterile conditions and were placed in phosphate-buffered saline (PBS) supplemented with 0.6% glucose and dissected to remove choroid plexus and aponevrosis. The cerebella were then cut into small pieces and collected in culture medium containing Dulbecco's modified Eagle's medium (DMEM-

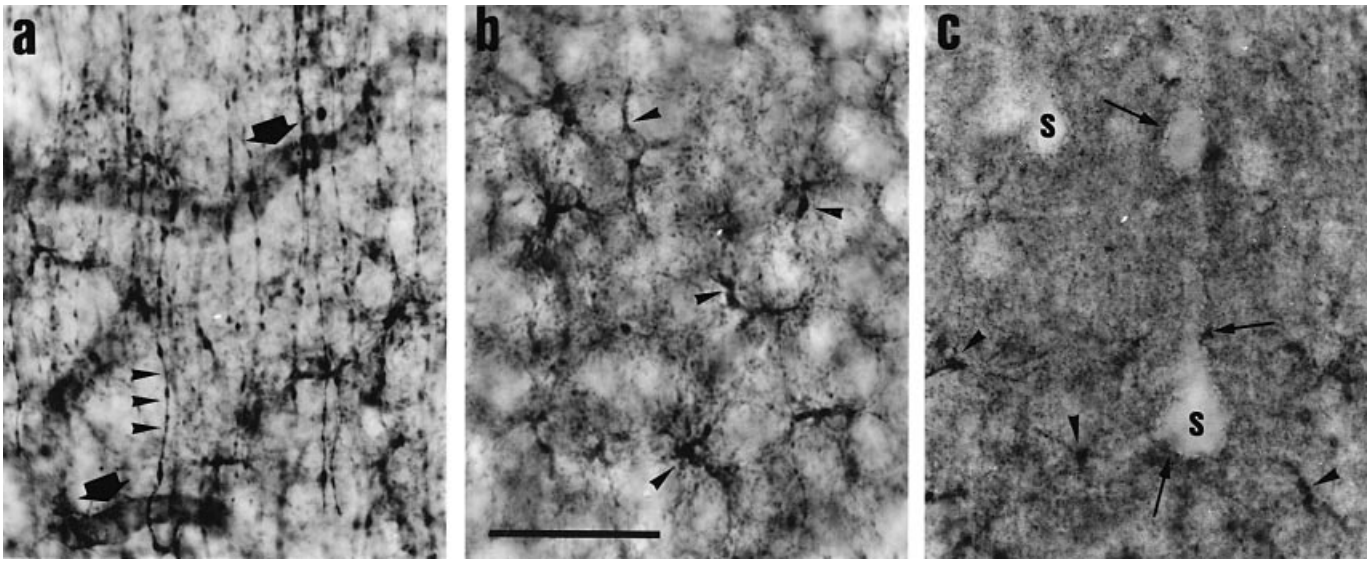


FIG. 3. Localization of acidic calponin during postnatal development of the cortex. Acidic calponin-IR is revealed with the peroxydase procedure. At P4 (a), typical extensions of radial glial cells are intensely stained (arrowheads). Blood vessels are also well stained (large arrows). At P10 (b), astrocytes processes are strongly stained in different cortical layers (arrowheads). In the adult cortex (c), acidic calponin-IR is diffusely distributed. Astrocytes are strongly stained (arrowheads), neuronal cell bodies (s) are not stained, but fine staining in the submembranous fields of the somatodendritic domains of pyramidal cells (arrows) can be seen. Bar, 50 µm.

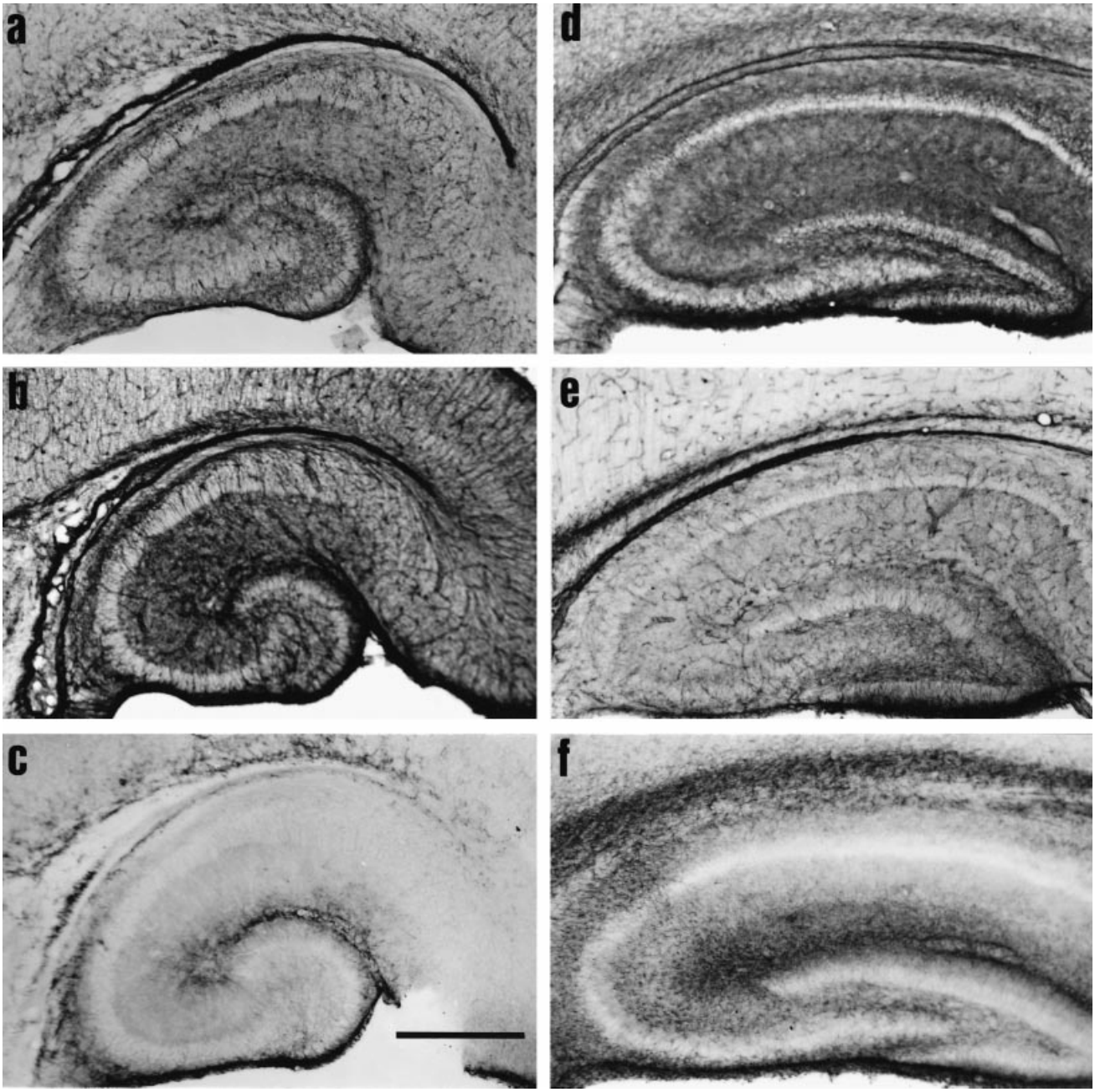


FIG. 4. Distribution of acidic calponin (a and d), vimentin (b and e) and GFAP (c and f) in serial sections of the hippocampal formation at P4 (a, b and c) and P10 (d, e and f). Immunoreactivities are revealed with the peroxydase procedure. Note the similar pattern of acidic calponin (a) and vimentin (b) immunoreactivities at P4, reflecting a predominantly radial glial staining. At this stage, GFAP-IR is low and glia limitans is preferently stained (c). At P10, acidic calponin-IR is characterized by a widespread distribution (d), whereas vimentin-IR (e) decreases and GFAP-IR (f) increases. Numerous astrocytes are stained. Neuronal cell body layers are not stained. Bar, 500 µm.

F12 from Gibco; 1 : 1) and supplemented with glucose (33 mM, Gibco), L-glutamin (2 mM, Gibco), $NaHCO_3$ (3 mM, Gibco), Hepes (5 mM, pH 7.4, Gibco) and insulin (25 µg/mL, Sigma). The cells were dissociated with a Pasteur pipette with a flame-narrowed tip, centrifuged at 500 ***g*** for 5 min, resuspended in the same culture medium supplemented with 5% Nu Serum (Becton Dickinson) and plated on poly ornithin (15 µg/mL, Mw 40 000, Sigma) coated coverslips to a density of 5×10^4 cells/ml. The cultures were maintained at 37 °C in a humidified 5% CO_2 : 95% O_2 atmosphere. The cells were washed with PBS and fixed with 4% PFA for 15 min after 1, 2, 3, 4 or 5 days of culture and then stored in PBS/Na-azide.

For cortical nerve cell cultures, neocortex was removed from E16 rat embryos, and neurons were developed in a chemically defined medium (Lafont *et al*., 1993) containing DMEM-F12, glucose (33 mM, Gibco), L-glutamin (2 mM, Gibco), $NaHCO_3$ (3 mM, Gibco), Hepes (5 mM, pH 7.4, Gibco) and supplemented with insulin (25 µg/mL, Sigma) transferrin (100 µg/mL, Sigma), progesterone (2.10^{-8}, Sigma), putrescin (6.10^{-5}, Sigma), selenium (3.10^{-8}, Gibco) and 0.1% ovalbumin (Sigma). Cortical cells were suspended on polyornithin-coated coverslips with no serum adjunction to a density of 8×10^4 cells/ml. Single and double immunofluorescence labelling were performed with cells cultured on coverslips; they were incubated

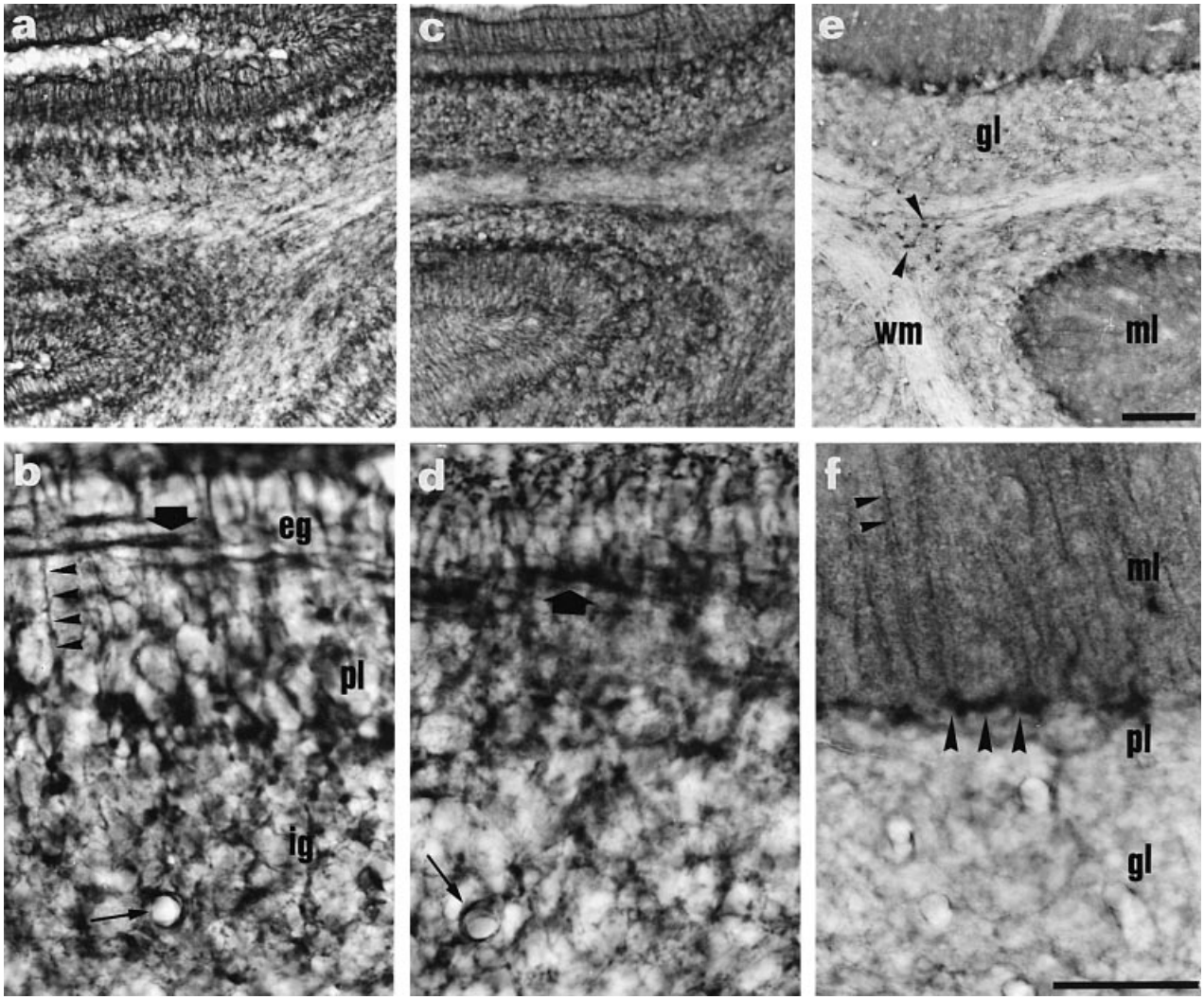


FIG. 5. Localization of acidic calponin during postnatal development of the cerebellum. Acidic calponin-IR is revealed with the peroxydase procedure. At P4 (a and b) and P10 (c and d) Bergmann glia (arrowheads in b) and granular cell layers (e.g. and ig) are intensely stained; blood vessels are also stained (arrows in b and d). Note the strong staining of the presumed parallel fibres (large arrows in b and d). In the adult cerebellum (e and f), Bergmann glia are still clearly immunoreactive (arrowheads in f). Acidic calponin-IR decreases in the granular layer (gl) and is diffusely distributed in the molecular layer (ml). Note the strongly immunoreactive astrocytes (arrowheads in e) in the white matter (wm). Pl, Purkinje cell layer. Bar, 100 µm (a, c and e) and 50 µm (b, d and f).

with primary antibodies for 24 h and with secondary antibodies conjugated with either rhodamine or fluorescein (Vector, 1 : 200) for 2 h or with phalloidin-rhodamine (Molecular Probes; 1 : 200) for 30 min. Double-labelled preparations were analysed with fluorescence microscopes (Nikon and Olympus).

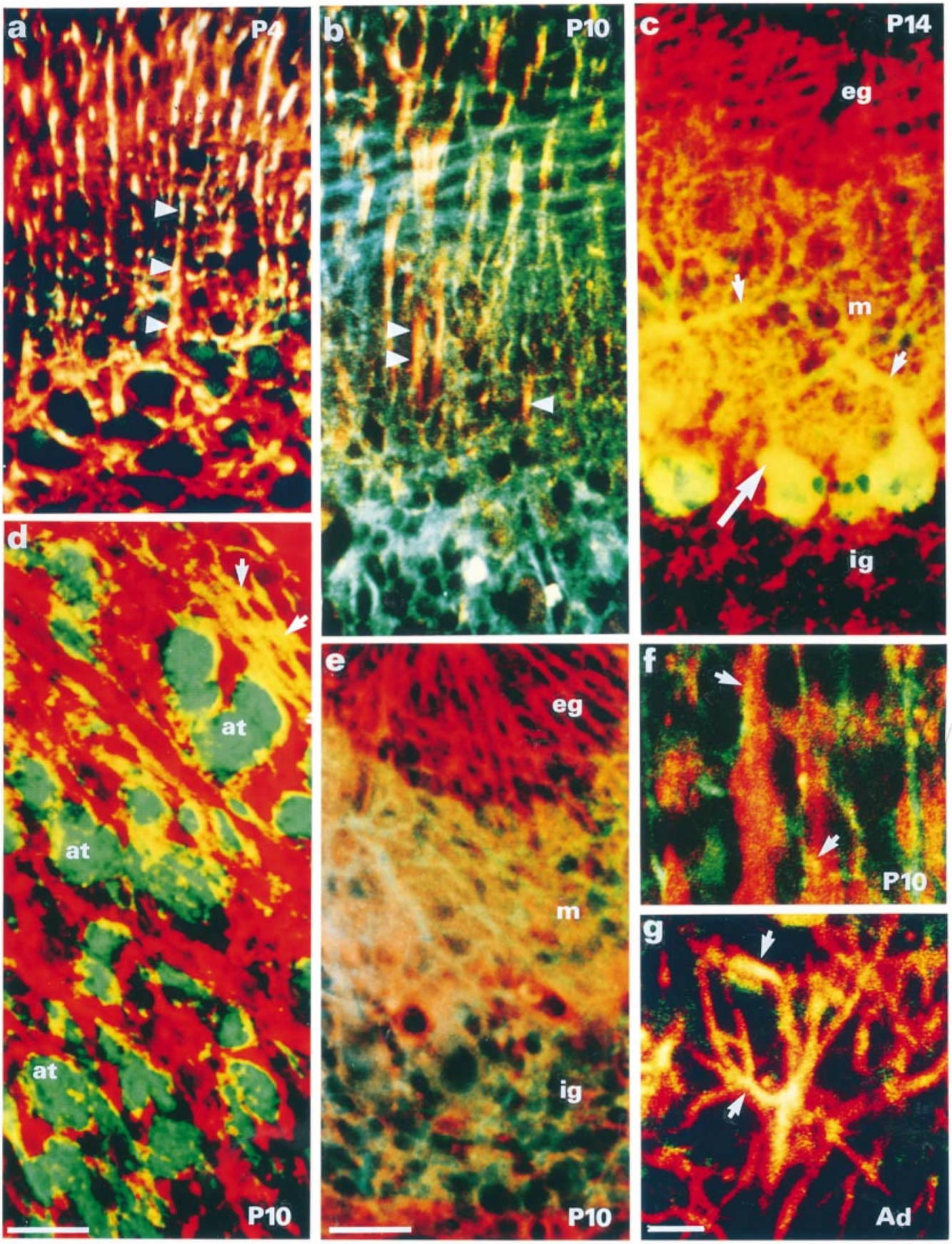
Results

Specificity of calponin antibodies used in this study and acidic calponin isoforms detected during the postnatal development of the rat brain

Immunoreactivity of the antibodies used in the present study was examined by immunoblotting. Reactivity of total cerebellum with polyclonal antibody to gizzard calponin has been previously reported (Represa *et al*., 1995; Trabelsi-Terzidis *et al*., 1995). The antibody we have raised against the acidic C-terminal sequence of the rat brain calponin RCp also stained a single band in the total homogenate of rat hippocampus with the same apparent molecular weight on SDS-gel (Mr) of 36 kDa (Fig. 1, lane 6 and 7), but it had no cross-reaction with the purified gizzard calponin (Fig. 1, lane 5). Throughout our experiments, no isoform of lower Mr was detected. However, during postnatal rat brain development, two-dimensional gel patterns obtained under the conditions described in the Materials and methods section showed the presence of several acidic calponin isoforms: two to three well-defined spots were visualized in rat cerebellum and three to four in rat hippocampus (Fig. 2). In both cases, the expression of the more acidic isoform was down-regulated during postnatal development; this isoform is definitively lost at P10 in the hippocampus, but only transiently in the cerebellum (Fig. 2). Unlike smooth muscle tissues (Draeger *et al*., 1991), rat brain cerebellum and hippocampus do not seem to contain detectable amounts of isoforms of lower Mr or basic isoforms of calponin. Experiments carried out on the X-100 Triton insoluble cytoskeleton (data not shown) give evidence that these spots correspond to genuinely different forms and are not due to residual phosphorylation of the same isoform.

Regional and cellular distribution of acidic calponin during postnatal brain development

The analysis of postnatal sections of rat brain revealed that acidic calponin was widely distributed, although the immunoreactivity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pattern was developmentally regulated in a spatial and temporal manner. Based on anatomical and morphological criteria, we describe here the distribution of acidic calponin-IR in three large regions of the central nervous system, which were intensely stained (frontoparietal cortex, hippocampus and cerebellum) at three significant stages of postnatal development (P4, P10 and adult).

In the cortex of P4 rats a strong acidic calponin-IR was observed in both radial glial cells and large blood vessels (Fig. 3a). Glia limitans was also stained (not shown). Numerous acidic calponin positive astrocytes appeared in the different cortical layers from P7 to P10 (Fig. 3b) corresponding to the period of cellular transformation from radial glia into astrocytes. Neuronal staining was absent at birth and progressively increased in the dendrites from P10 to the adult (Fig. 3b and c). Neuronal cell bodies were not stained (Fig. 3c), though a fine staining of the submembranous area of mature pyramidal cells cannot be excluded.

In the hippocampal formation, the pattern of calponin-IR was similar to that of the neocortex with a predominant glial staining (glia limitans, radial glia and astrocytes). Acidic calponin-IR was then compared with vimentin (an intermediate filament protein which is mainly and strongly expressed by radial glia in the developing rat brain), and GFAP immunoreactivities on almost adjacent sections at P4 and P10. At P4, the acidic calponin (Fig. 4a) and vimentin (Fig. 4b) distributions were practically identical, whereas GFAP (Fig. 4c) was restricted around the hippocampal fissure, the fimbria and the corpus callosum. At P10, acidic calponin-IR (Fig. 4d) showed a more homogeneous and widespread distribution throughout the hippocampus, suggesting that acidic calponin was probably present in both astrocytes and neurons. In comparison, vimentin-IR (Fig. 4e) strongly decreased, whereas GFAP-IR (Fig. 4f) increased and stained numerous astrocytes, mainly in the CA3 region, but none of these immunoreactivities was identical to or completely different from acidic calponin-IR

In the cerebellum of rat pups (P4 and P10), acidic calponin-IR was observed in Bergmann-radial glial cells and in blood vessels (Fig. 5a and b). Neuronal staining was also observed in the internal and external granular cell layers (Fig. 5a, b, c and d) where the antibody apparently stained parallel fibres and granule cells, in contrast with the Purkinje cell bodies, which were not stained (Fig. 5b and d). From P10 (Fig. 5c and d) to adult (Fig. 5e and f), acidic calponin-IR decreased in the granular cells but diffusely increased in the molecular layer (Fig. 5f). Bergmann glia were still clearly immunoreactive (Fig. 5f) and astroglial cells were also strongly stained in the white matter (Fig. 5e).

TABLE 1. Acidic calponin in postnatal rat brain *in vivo*

	Localization *in vivo* (developing rat brain)		
	P4	P10	Adult
Cortex			
Pyramidal cells (s)	–	+/–	+/–
Pyramidal cells (d)	–	+/–	+
Radial glia	+++	+[(a)]	
Astrocytes	+	++	++
Hippocampus			
Pyramidal cells (s)	–	+/–	+
Pyramidal cells (d)	–	+	+
Granular cells (s)	–	–	–
Granular cells (d)	–	+/–	+/–
Radial glia	+++	++	+[(b)]
Astrocytes	+	++	++
Cerebellum			
Purkinje cells (s)	–	+/–	+/–
Purkinje cells (d)	–	+/–	+
External granular cells	++	++	
Internal granular cells	++	+	+/–
Bergmann glial cells	+++	+++	++
Astrocytes	+	++	++
Choroid plexus	+++	+++	+++
White matter[(c)]	–	–	–
Blood vessels:	+++	++	++

For all neurons analysed here, immunolocalization was measured in the dendritic (d) and somatic (s) fields. [(a)]At P10, few radial fibres were still detected in the different cortical layers. These radial fibres disappear in the adult cortex. [(b)]Radial-like glial cells in the granular layer of the dentate gyrus. [(c)]White matter corresponds to corpus callosum, axonal tracts in the striatum and the cerebellum and fimbria in the hippocampus. Immunostaining: –, no staining; +/–, very low or heterogeneous staining showing only some immunopositive cells; +, low staining; ++, medium staining; +++, strong staining.

Colocalization of acidic calponin with different glial and neuronal markers in vivo

In order to confirm the glial and neuronal localization of acidic calponin during the postnatal development of the rat brain, double immunofluorescence staining experiments were done and analysed with a LASER scanning confocal microscope. The different markers used were vimentin, GFAP (see above), beta III tubulin and MAP-2 (neuronal differentiation markers which are components of brain microtubules) and parvalbumin (a calcium-binding protein which is a relatively specific Purkinje cell marker in the cerebellum).

Vimentin was strongly colocalized with acidic calponin in the radial glial cells of the cortex, hippocampus and cerebellum in the first days of postnatal development. For instance, this colocalization is illustrated at P4 in cerebellar Bergmann glial fibres and also around Purkinje cell bodies in glial cell processes (Fig. 6a). GFAP was also colocalized with acidic calponin in the glia limitans, the radial glial cells and the astrocytes. This accurate glial colocalization was also observed in the Bergmann glial fibres

FIG. 6. Double immunostaining depicting the colocalization of acidic calponin with glial and neuronal markers in different regions of the postnatal rat brain. In the present digitized confocal images, 'pseudocolors' have been used to depict rhodamine (in red), fluorescein (in green) and the colocalization of both fluorochromes (in yellow). (a) Acidic calponin-IR (red) and vimentin-IR (green) in the cerebellum at P4 (10-μm optical slices). Both proteins are strongly colocalized in Bergmann fibres (arrowheads). (b) Acidic calponin-IR (green) and GFAP-IR (red) in the cerebellum at P10 (10-μm optical slices). Both proteins are colocalized in Bergmann fibres (arrowheads). (c) Acidic calponin-IR (red) and parvalbumin-IR (green) in the cerebellum at P14 (10-μm optical slices). At this developmental stage, acidic calponin is clearly present in Purkinje cell bodies (large arrow) and dendrites (small arrows). (d) Acidic calponin-IR (red) and beta-III-tubulin-IR (green) in the striatum at P10 (15-μm optical slices). Colocalization of both proteins can be observed (arrows) but axonal tracks (at), strongly immunoreactive for beta-III-tubulin, are devoid of acidic calponin-IR. (e) Acidic calponin-IR (red) and MAP-2-IR (green) in the cerebellum at P10 (10-μm optical slices). Both proteins are present in the molecular layer (m) and in the internal granular cell layer (ig). MAP-2 is absent from the immature external granular cell layer (for example). (f) Acidic calponin-IR (green) and MAP-2-IR (red) in the neocortex at P10 (5-μm optical slices). At this developmental stage, acidic calponin is present with MAP-2 in the dendritic shafts (arrows). (g) Acidic calponin-IR (green) and GFAP-IR (red) in the adult cortex (5-μm optical slices). Both proteins strongly colocalized in astrocytes (arrows). Bar, 20 μm (a, b, c and e); 10 μm i (f and g) and 45 μm (d).

at P10 in the cerebellum (Fig. 6b) and in numerous astrocytes of the adult rat (Fig. 6g). Double immunostaining of acidic calponin with beta III tubulin clearly show that acidic calponin was present in some neuronal elements, but seemed to be virtually absent from axonal tracts such as the striatal axonal bundles in both pups (Fig. 6d) and adult rats. In rat pups, MAP-2 colocalization also

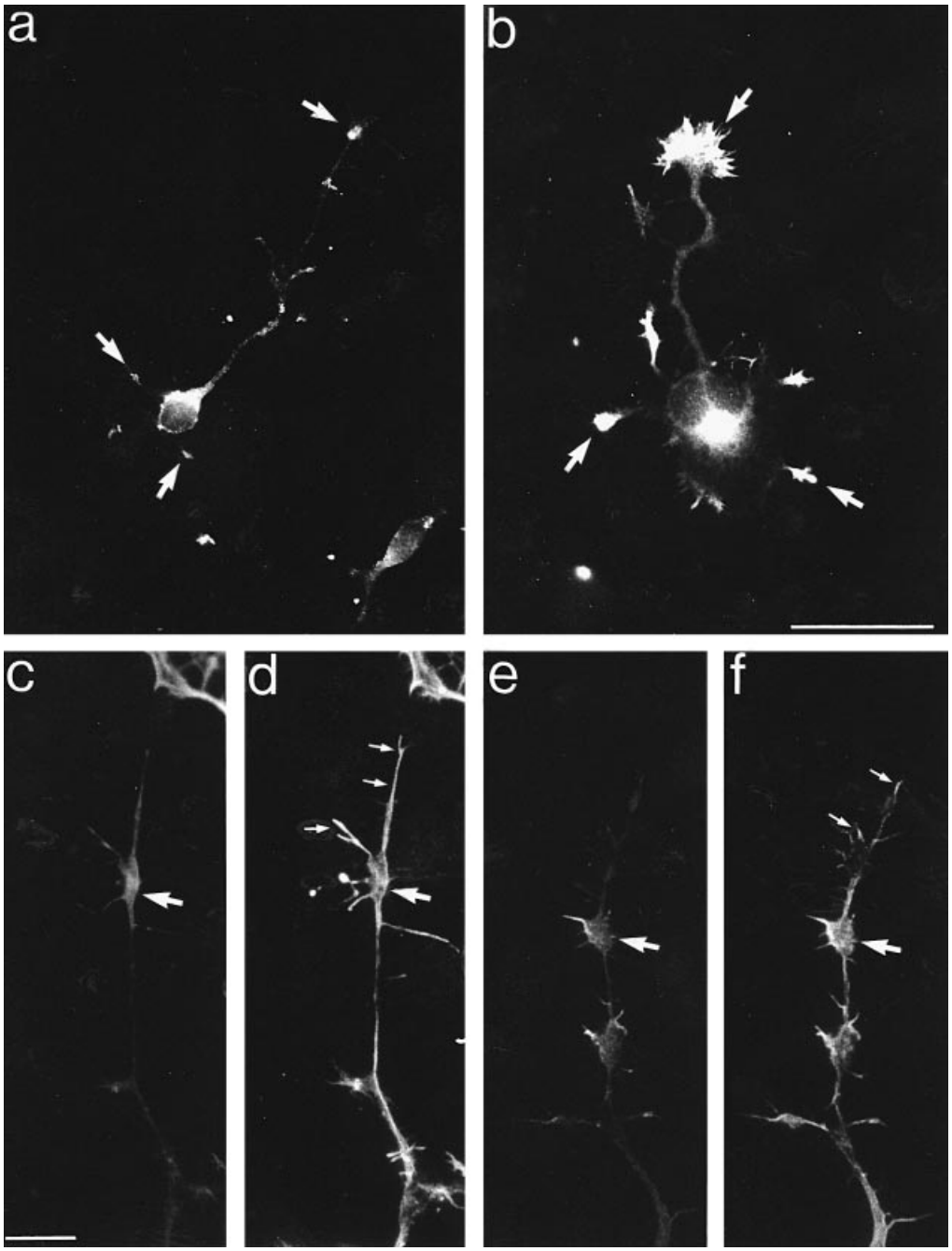
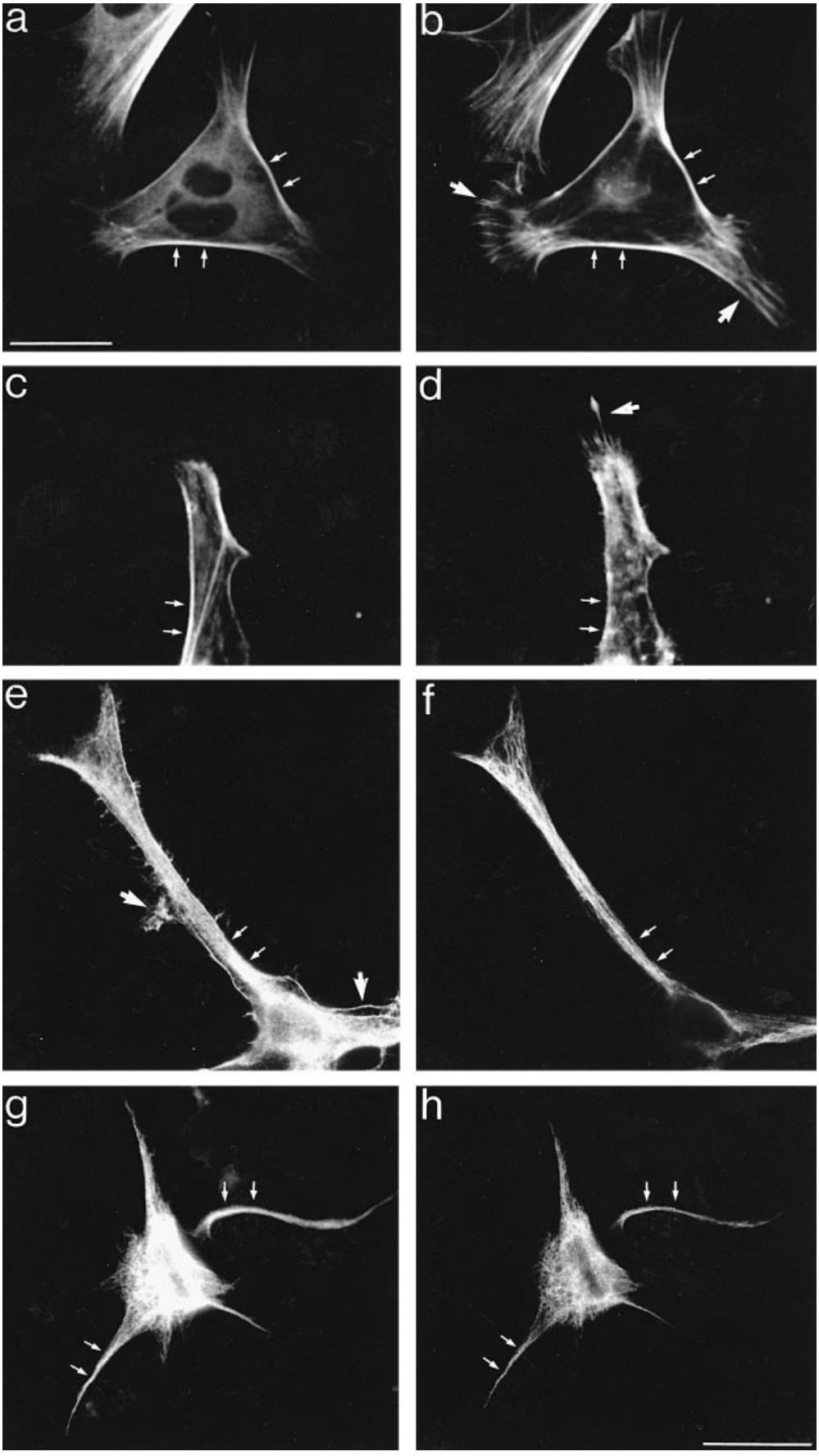


FIG. 7. Localization of acidic calponin in neuronal cultured cerebellar granule cells and cortical cells. Acidic calponin immunoreactivity is revealed with fluorescein conjugated secondary antibodies (a, b, c, and e) and actin filaments are revealed with phalloidin-rhodamine (d and f). Acidic calponin is enriched in the growing tips of granular cells (a: large arrows) and cortical cells (b: large arrows). For instance, note the typical well-spread axonal growth cone in (b). Double staining with phalloidin depicts a strong colocalization of acidic calponin and actin filaments in the core of the growth cones (c–f: large arrows). Nevertheless, actin filaments are not saturated with acidic calponin and many filopodia are devoid of the protein (d and f: small arrows). Bar, 30 µm (a and b); 10 µm (c–f).

FIG. 8. Localization of acidic calponin in cultured cerebellar glial cells. Immunoreactivities are revealed with fluorescein and rhodamine conjugated secondary antibodies and actin filaments are revealed with phalloidin-rhodamine. (a, c, e and g) Acidic calponin-IR. (b and d) Colabelling with phalloidin. (f) GFAP-IR. (h) Vimentin-IR. Acidic calponin is enriched at the periphery of glial cells, in stress fibres, in the submembranous fields (a and c: small arrows) and in the growing tips (c and g: small arrows). In these cases, although the colocalization with F-actin is obvious (b and d: small arrows), phalloidin stained protrusions are not stained with antiacidic calponin antibodies (b and d: large arrows). Acidic calponin is also present in gliofilaments domains (f and h: small arrows). Bar, 25 µm.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demonstrated that acidic calponin was present in neurons and, for example, in the differentiated dendrites of the granular and Purkinje cells in the cerebellum (Fig. 6e) and in the dendritic shaft of the pyramidal cells in the cortex (Fig. 6f). In order to confirm the localization of acidic calponin in Purkinje cells, calponin antibodies were combined with parvalbumin antibodies. The colocalization of both IRs was obvious at P10 but not before. At P14, parvalbumin and acidic calponin were clearly colocalized in the somatodendritic fields of all Purkinje cells in the cerebellum (Fig. 6c); a few Purkinje cell axons were also immunoreactive (not shown). Table 1 summarizes the findings on the localization of acidic calponin in the developing rat brain.

Acidic calponin is particularly enriched in growth cones, beneath the plasma membrane, and along actin and intermediate filaments (GFAP and vimentin) of cultured brain cells

The localization of acidic calponin in developing neurons such as granule cells and their eventual presence in parallel fibres and Purkinje cell axons, supported the notion that this protein may be involved in the process of neurite outgrowth. For this reason, the presence of acidic calponin in cultured cerebellar cells from P2 to P4 and cortical neurons from E16 was investigated. The cellular density used was relatively low in order to observe the morphological features of growing cells and in particular growing tips such as growth cones.

Cultured cells were intensely stained with polyclonal antiacidic calponin antibodies (Figs 7 and 8). In general, the strongest staining was seen at the periphery and at the tips of the growing cells. In the cell body, the immunoreactivity appeared to be restricted to the perinuclear area. In neurons (Fig. 7), the pattern of acidic calponin-IR was similar in cerebellar (Fig. 7a) and cortical cells (Fig. 7b). Acidic calponin was particularly enriched in the core of the growth cones.

Phalloidin-rhodamine double staining was performed in order to investigate the acidic calponin localization on the F-actin domains. In neuronal growth cones (Fig. 7c–f), protrusive filopodial extensions, strongly labelled with phalloidin-rhodamine, were not acidic calponin immunopositive in their extremity. In astroglial cells, clearly identified with anti-GFAP antibodies, acidic calponin-IR was observed in the actin stress fibres and in the cell membrane (Fig. 8a), in the glial growing tips (Fig. 8c) and in some short protrusions that arise from the astroglial extensions (Fig. 8e). In these cases, actin filaments were not saturated with acidic calponin, and many filopodial extensions were devoid of this actin binding protein (Fig. 8a–d).

TABLE 2. Acidic calponin in postnatal rat brain *in vitro*

	Localization *in vitro* (growing cell cultures)	
	Astroglial cells ($GFAP^+$)	Neuronal cells (β III $tubulin^+$)
Soma	+	+
perinuclear	++	++
nucleus	–	–
Glial extentions	++	
Neurites		+/–
Growth cones and tips	+++	+++
Cell cortex	+++	++
Protrusions	+/–	+/–

Immunostaining: –, no staining; +/–, alternative or heterogeneous staining; +, low staining; ++, medium staining; +++, strong staining.

Growing astrocytes were also strongly labelled in regional domains of gliofilaments (Fig. 8e–h). For instance, acidic calponin and vimentin were strikingly colocalized in a large number of cells and especially along the extending processes (Fig. 8g and h). Cell nuclei of neuronal, glial and fibroblastic cells were never stained with antibodies against acidic calponin. Table 2 summarizes the findings on the localization of acidic calponin in cerebellar and cortical cell cultures.

Discussion

In the present report we show for the first time that acidic calponin is particularly enriched in the growth cones where it codistributes with microfilaments. These results, in agreement with previous observations depicting a higher expression of acidic calponin mRNA in maturing neurons (Ferhat *et al.*, 1996) suggest a role for calponin in the processes of neurite outgrowth and navigation. We further confirm that acidic calponin is present in glial cells during the postnatal development and is mainly observed in radial glia, Bergmann glia, glia limitans and mature astrocytes. Our data show that acidic calponin colocalizes in these cells not only with microfilaments but also along glial intermediate filaments (vimentin and GFAP), suggesting that this actin-binding protein may interact with other cytoskeletal components. Finally, we also found that acidic calponin has a group of three to four isotypes which are down-regulated during the postnatal development of the rat brain. These points are discussed below.

Acidic calponin isoforms

Two-dimensional gel electrophoreses show the presence of three (in the cerebellum) and four (in the hippocampus) different subisoforms of acidic calponin in maturing brain. Nevertheless, according to Ferhat *et al.* (1996), only one single acidic calponin mRNA could be detected in adult or developing rat brains and cultures. Consequently, these subtypes probably correspond to post-translational modifications. Though we cannot exclude the possibility that these isoforms may or may not be phosphorylated, the respective pI values seem incompatible with the idea that they represent different phosphorylation states of the same protein. Whatever the nature of the post-translational modifications of acidic calponin, it is worth noting that in the hippocampus, the more acidic isoform is apparently lost during postnatal maturation, while in the cerebellum the more acidic form is transiently down-regulated by P10. Draeger *et al.* (1991) have also reported a variability in the expression of basic calponin in smooth muscle during development. However, these authors found higher basic calponin-IR in the more mature smooth muscle. Altogether, these data suggest a complex and fine regulation of acidic calponin depending on the age and the structure considered. These structural modifications may eventually influence the roles of calponin by changing, for example, its binding activity either to actin or to other cytoskeletal proteins. Further analyses are required to clarify these points.

Potential role of acidic calponin in neuronal cells

Previous reports from our laboratory (Represa *et al.*, 1995; Trabelsi-Terzidis *et al.*, 1995), using antibodies against the basic domain of calponin, depicted a preferential staining of the somatodendritic compartments of mature neurons. Preliminary electron microscopy data (C. Agassandian, M. Plantier, A. Fattoum, I. Jorquera, Y. Ben-Ari, A. Represa and E. der Terrossian, unpublished observations) confirmed these observations and additionally demonstrated the preferential localization of acidic calponin to dendritic spines,

including the postsynaptic density. We have therefore suggested (Represa *et al*., 1995) that acidic calponin may play a role in the morphological changes associated with synaptic plasticity. In mature CNS, acidic calponin is clearly absent from axonal pathways, but the results presented here show that immature axons are stained by calponin antibodies *ex vivo* (growth cones and axon shafts of cerebellar granule cells and cortical neurons) and *in vivo* (mainly the axons of cerebellar granule cells, but also a few Purkinje cell axons). The presence of acidic calponin in the growing tips of brain cells suggests that this protein may modulate the actin-based guidance of these structures. The organization of growth cone actin and myosin network has been well described using light and electron microscopy (Kuczmarski & Rosenbaum, 1979; Bridgman & Dailey, 1989; Lewis & Bridgman, 1992), and numerous links have emerged on the functional role of the actomyosin system in growth cones (Forsher & Smith, 1988; Suter & Forsher, 1998). For instance, Lin *et al*. (1996) have demonstrated that actomyosin inhibitors such as 2,3-butane-dione-2-monoxime (BDM) and N-ethylmaleimide (NEM) -inactivated myosin S1 fragments attenuate the retrograde F-actin flow in neuronal growth cones in a dose-dependent manner. Furthermore, inhibition of actin-activated myosin Mg^{2+}-ATPase activity (Shirinsky *et al*., 1992), stabilization of the actin microfilaments (Kake *et al*., 1995) and/or bundling of these filaments (Kolakowski *et al*., 1995; Tang & Janmey, 1996) by calponin have been demonstrated *in vitro*. All these observations suggest that acidic calponin may contribute to the modulation of the length and size of the lamellofilopodial structure of growth cones. Although the present study shows a good colocalization of acidic calponin with phalloidin-rhodamine in growth cones, there are clearly some actin filaments in both cytoplasm and filopodia which are devoid of acidic calponin-IR. This may indicate that acidic calponin has a saturated capacity to bind actin filaments or that other related proteins such as caldesmon (Sobue, 1993; Kira *et al*., 1995; Represa *et al*., 1995) may contribute to the regulation of the actomyosin system in neuronal cells. In fact, though acidic calponin seems to be quite widely expressed in the brain, it is particularly enriched in the cerebellum and in the olfactory bulb of developing rats and moderately expressed by hippocampal and cortical cells (also see Ferhat *et al*., 1996). The remaining brain areas are only poorly stained by calponin antibodies and probes, emphasizing the need for other actomyosin regulatory proteins in these brain areas.

In the present study, the analysis of acidic calponin-IR suggests that immunopositive cells are postmitotic differentiating neurons. Interestingly, adenovirus-mediated transfer of the smooth muscle cell calponin gene has been found to be associated with inhibition of the proliferation of smooth muscle cells and fibroblasts and may result from inhibition of actomyosin interactions underlying cytokinesis (Jiang *et al*., 1997). These observations are consistent with the idea that acidic calponin could play a role in stopping the proliferation of neuronal precursors and contribute to their differentiation.

Potential role of acidic calponin in glial cells

The presence of acidic calponin in radial glia, astrocytes and, particularly, in the elongating glial processes known to be highly motile (they rapidly extend microspikes and lamellopodia) suggests that the mechanisms presently evoked for neuronal growth cones or neuronal differentiation (see above) can also be important in glial cells. One typical difference between the growing glial and neuronal processes is the presence and the abundance of intermediate filaments in the core of the extending glial tips (Mason *et al*., 1988). Astroglial filaments are composed of intermediate filament protein subunits, GFAP and vimentin. Strong colocalization of acidic calponin with GFAP and vimentin was observed along the extensions and at the growing tips of astrocytes but was absent in mature astrocytes in culture (not shown). This demonstrates that acidic calponin is as much present in gliofilament domains as in actin filament domains. Interaction and association of smooth muscle calponin with intermediate filaments such as desmin has been reported (Wang & Gusev, 1996; Mabuchi *et al*., 1997). All these data are consistent with the possible cytoskeletal role of calponin in the organization of both actin and intermediate filaments. Nevertheless, in our hands, colabelling assays of acidic calponin with light and heavy subunits of neurofilament proteins (NF-L and NF-H) show that there is no obvious relationship between acidic calponin and the neuronal intermediate filaments (data not shown). Moreover, in agreement with its potential association with the plasma membrane and its interaction with phospholipids *in vitro* (Fujii *et al*., 1995), the presence of acidic calponin beneath the plasma membrane and in the cortex of brain cells seems to suggest that it plays a determinant role in cell morphology by interacting with some essential cytoskeletal components.

In conclusion, we raised a specific polyclonal antibody against acidic brain calponin and analysed the developmental changes in expression and localization of this protein in the developing rat CNS. We showed that acidic calponin has a group of different subisoforms, probably generated after synthesis, which are down-regulated during maturation. The strongest immunoreactivity is observed in radial glia, mature astrocytes and postmitotic neurons. The enrichment of acidic calponin immunoreactivity in growth cones is compatible with the hypothesis that acidic calponin contributes to the regulation of the motility of neural cells. Its striking colocalization with cytoskeletal filaments such as F-actin, vimentin and GFAP suggests that acidic calponin may also play a role in cell tension and cell morphology.

Acknowledgements

Special thanks to Dr I. Lebbar for the purification of rat brain actin, to Dr A. Chavanieu for his help in preparing the synthetic peptide, to J. P. Pagnac for photographic assistance and to I. Jorquera for expert technical assistance. This work was supported by a grant from the Institut National de la Santé et de la Recherche Médicale (INSERM).

Abbreviations

EGTA, ethylen glycol tetraammonium; GCp, gizzard calponin; GFAP, glial fibrillary acidic protein; HPLC, high-performance liquid chromatography; IEF, isoelectric focusing; IR, immunoreactivity; MAP-2, microtubule-associated protein 2; Mr, apparent molecular weight on SDS-gel; Mw, molecular weight; PAGE, polyacrilamid gel electrophoresis; pI, isoelectric point; RCp, rat calponin; RT-PCR, reverse transcriptase polymerase chain reaction; SDS, sodium dodecyl sulphate.

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