Expression of an acidic isoform of calponin in rat brain: Western blots on one- or two-dimensional gels and immunolocalization in cultured cells

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Calponin, an actin- and Ca^{2+} -calmodulin-binding protein characterized as an inhibitory factor of the smooth-muscle actomyosin activity, has also been shown to be present in some non-muscle cells. However, there is a controversy as to whether calponin is present or not in brain. Several laboratories indicate that this protein is absent in chicken or bovine brains, while Applegate et al. [Applegate, Feng, Green and Taubman (1994) *J. Biol. Chem.* **269**, 10683–10690] have recently reported the identification of an mRNA specific for a 36 kDa non-muscle calponin analogue in homogenates of rat brains. For the first time we demonstrate, by Western blots and *in situ* immunofluorescence localization using

monoclonal as well as affinity-purified polyclonal antibody to gizzard calponin, that a 36–37 kDa and a 35–36 kDa calponin-like proteins are expressed respectively in pig and rat brains and in rat cerebellar cultured cells. The acidic pI (5.2–5.4) of the rat brain protein revealed by isoelectric focusing is in good agreement with that of the protein coded for by the calponin isoform mRNA described by Applegate et al. and is different from that of the protein from chicken gizzard (pI 9.9). Brain calponin-like protein is different from two other Ca^{2+} -calmodulin-binding proteins previously identified in brain, namely caldesmon and adducin, and from tropomyosin.

INTRODUCTION

Calponin was first discovered in 1986 by Takahashi's laboratory [1]. It was characterized as an actin-, tropomyosin- and Ca^{2+} -calmodulin-binding protein and has been purified from several vertebrate smooth-muscle tissues ([1,2; see also reviews in [3,4]). Recently mammalian calponins have been cloned and sequenced [5–7]. Like caldesmon, calponin has been described as an inhibitory factor of the actin-activated Mg^{2+} -ATPase, providing growing evidence that this protein is part of the regulatory machinery in smooth-muscle cells [3,8,9]. Calponin is heat-stable, very sensitive to proteolysis, and presents several isoforms. Although this protein is still regarded as specific for smooth-muscle cells by some workers, evidence has been found for the presence and the localization of calponin-immunoreactive proteins in the actin cytoskeleton of several non-muscle cells such as mouse fibroblasts, bovine adrenal gland and platelets by Takahashi and collaborators [2,11]. Surprisingly, the same group has also reported that chicken as well as bovine brains do not contain calponin [2,5]. However, in a very recent paper, Applegate and co-workers [10] have identified an mRNA specific for a 36 kDa non-muscle calponin analogue in homogenates of rat brain.

In the present paper we provide evidence for the presence of a calponin-immunoreactive protein expressed in rat and pig brain tissues and in rat cerebellar cultured cells.

MATERIALS AND METHODS

Primary cultures

Rat cerebellar neurons were prepared as described previously [12]. In brief, cerebella were taken from brains of 8-day-old rats

using 0.1 % trypsin/0.1 % DNAase for cell dispersion. Cultures were maintained in Dulbecco's modified Eagle's medium (DMEM)/Hams F12 medium containing 10 % (v/v) fetal-calf serum at 37 °C under an atmosphere of humidified CO_2 /air (1:19). Cultures were used after 11 days *in vitro*. Cerebellar glial cells were prepared as previously outlined [13].

Animals

Pig brain was obtained from the local slaughterhouse and stored at -80°C . Male rats (Wistar; 150–200 g body wt.; 2 months old) were killed by intraperitoneal injection of a lethal dose of sodium pentobarbital. The chest cavity was opened, and the animal's heart was perfused with 0.9 % NaCl to remove blood. Freshly dissected brain cortex, hippocampus, cerebellum and trachea were washed with 150 mM NaCl/10 mM EGTA/1 mM NaN_3 /1 mM dithiothreitol (DTT)/0.5 mM phenylmethanesulphonyl fluoride (PMSF)/2 mM sodium tetrathionate/0.5 mM benzamidine/proteinase-inhibitor cocktail (10 $\mu\text{g}/\text{ml}$ each leupeptin, pepstatin, trypsin inhibitor and chymostatin). Tissues were immediately incubated in a boiling-water bath for 2 min and chilled in an ice bucket. All subsequent procedures were carried out at 4 °C.

Preparation of proteins

All extracting buffers contained 10 mM EGTA, 2.5 mM EDTA, 1 mM DTT, 0.2 mM PMSF, 1 mM NaN_3 , 0.5 mM benzamidine, 2 mM sodium tetrathionate, 0.5 mM di-isopropyl fluorophosphate (DFP) and 60 $\mu\text{g}/\text{ml}$ proteinase-inhibitor cocktail. In subsequent steps the concentration of the proteinase-inhibitor cocktail was lowered to 2 $\mu\text{g}/\text{ml}$ and DFP was omitted.

Turkey gizzard calponin was purified as described by Takahashi et al. [1]. Partially purified pig erythrocyte adducin

Abbreviations used: DFP, di-isopropyl fluorophosphate; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; GFAP, glial fibrillary acidic protein; MAP2, microtubule-associated protein 2; PMSF, phenylmethanesulphonyl fluoride.

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and purified pig brain tropomyosin were prepared as in [14,15]. Protein concentrations were determined using a modification of the method of Bradford [16].

Primary antibodies

All polyclonal antibodies were raised in rabbit and affinity-purified on their respective antigen: antibodies to pig platelet caldesmon or to tropomyosin as in [3] to gizzard calponin as in [17]. Polyclonal anti- γ -actin was generously given by Dr. I. Lebbar (Paris). The specificity of each antibody was established by the fact that only their respective antigen was recognized in total tissue homogenates after fractionation of the proteins by SDS/PAGE followed by Western blot analysis.

Monoclonal antibodies to actin (clone KJ 43A), to MAP2 (clone HM-2) and to gizzard calponin (clone CP-93) were purchased from Sigma. Polyclonal antibody to glial fibrillary acidic protein (GFAP) was from Dako.

Gel electrophoresis and immunoblotting

SDS/PAGE was carried out in 10%-(w/v)-polyacrylamide slab gels by the method of Laemmli [19]. Proteins were identified either by Coomassie Blue staining or by Western blotting [20] on Immobilon [poly(vinylidene difluoride)] membrane having a pore size of $0.45\ \mu\text{m}$ (Millipore) for 15 h at 30 V in 20 mM Tris/150 mM glycine and 20% (v/v) methanol. Membranes were blocked with 0.5% (w/v) gelatin in PBS/0.2% Triton X-100.

Two-dimensional electrophoresis

Two-dimensional isoelectric focusing/PAGE was performed as described by O'Farrel [21]. Isoelectric focusing in the first direction was carried out in 4% polyacrylamide gel. Second-dimension PAGE was performed as described by Laemmli [19] on 0.1% SDS/10% polyacrylamide gels. The proteins were then either revealed by silver staining [22] or immunodetected using the enhanced chemiluminescence (ECL; Amersham) or the alkaline phosphate technique after electrotransfer on to Immobilon membrane as described above.

Immunocytochemical staining

Immunocytochemical procedures were assayed on both cultured glial cells or in neurons from rat cerebellum. Cells were cultured on coverslips and fixed after 11 days *in vitro* with 4% para-formaldehyde. Cells were preincubated with 0.2% Triton X-100/0.2% gelatin/10% normal goat serum/phosphate buffer, pH 7.4, and then with primary monoclonal or polyclonal antibodies overnight at room temperature.

RESULTS AND DISCUSSION

Calponin-immunoreactive bands in extracts at pH 8 or pH 6.9

Proteins were extracted from rat brain cortex at pH 6.9 as described by Takahashi et al. [1] or at pH 8 by the method of Yamashiro-Matsumura and Matsumura [18] (including the heat-treatment step). They gave different patterns of calponin-immunoreactive bands when analysed by Western blots, using monoclonal antibody to gizzard calponin. At pH 8, only one single band of about 70 kDa was revealed, whereas at pH 6.9 two bands of 70 kDa and 97 kDa were detected (Figure 1). Since calponin from smooth-muscle cells and its isoform described by Applegate et al. [10] contain respectively two [4,6] and three [10] thiol groups, our results suggested that disulphide bonds might be formed during extraction, although all the buffers we used

Figure 1 Western-blot analysis with monoclonal antibody to calponin

(a) Coomassie Blue-stained gels of 100 μg proteins from rat brain cortex homogenized in 3.5 vol. of either 20 mM Tris/HCl/100 mM NaCl, pH 8 (lane 2) or 300 mM KCl/50 mM imidazole, pH 6.9 (lane 3). Lane 1, Pharmacia standards of low molecular mass (M). (b) Blots of parallel samples, at pH 8 (lane 4) and at pH 6.9 (lane 5), labelled with the monoclonal antibody to calponin (1:2000). Immunoreactive bands (one single band in lane 4 and two bands in lane 5) were revealed with peroxidase-streptavidin conjugate (1:1000; Vector) after incubation with biotinylated rabbit anti-mouse IgG (1:400; Dako).

contained 1 mM DTT. We speculate that, since the pK for these groups is 8.0–8.6, the cross-reaction leading to the formation of disulphide bonds would be very fast and complete at pH 8, and that the slower reaction at pH 6.9 probably allows the formation of some dimers, but also of trimers. We therefore checked this hypothesis by performing Western-blot analysis on rat cerebellum directly treated with the Laemmli denaturing buffer, without any previous extraction of proteins. Under these conditions, decoration with the monoclonal antibody to gizzard calponin showed a strong staining of a 34 kDa and a less reactive 70 kDa protein (Figure 2, lane 4). The same pattern was obtained with the affinity-purified polyclonal antibody to gizzard calponin, confirming the specificities of both antibodies. In order to effect

Figure 2 Immunoblot analysis of calponin-like proteins from rat cerebellum and trachea

Cerebellum and trachea were directly solubilized in the Laemmli sample buffer. (a) SDS/PAGE and Coomassie Blue stain. (b) Immunoblots of parallel aliquots were immunostained with monoclonal antibody to calponin as described in Figure 1. Lanes 1 and 4, rat cerebellum (40 μg of proteins); lanes 2 and 5, rat smooth-muscle trachea (15 μg of proteins); lane 3, molecular-mass (M) markers.

Figure 3 The calponin-like protein detected in pig brain cortex is different from tropomyosin and adducin

(a) SDS/PAGE and Coomassie blue stain. (b) Immunoblots. Lane 1, molecular-mass (M) markers; lanes 2 and 4, 50 μ g of 45% $(\text{NH}_4)_2\text{SO}_4$ precipitate from pig brain cortex extracted proteins in buffer B; lanes 3, 6 and 7, 4 μ g of purified pig brain tropomyosin; lane 5, 0.5 μ g of purified gizzard calponin. Antibodies used were polyclonal antibodies to gizzard calponin at 2 μ g/ml (lanes 4–6) and to pig platelet tropomyosin at 1 μ g/ml (lane 7). (c) Lane 8, molecular-mass markers; lane 9, Coomassie Blue-stained gel of partially purified erythrocyte adducin (*); lane 10, the corresponding immunoblot using the polyclonal antibody to gizzard calponin.

Figure 4 Expression of the calponin-like proteins in neurons and in glial cells cultured from rat cerebellum

(a) Lanes 1 and 2, Coomassie Blue-stained gels of homogenates from cerebellar glial cells (lane 1) and neurons (lane 2); lane 3, molecular-mass (M) markers. (b) Immunoblots. Glial cells (lane 4), neurons (lane 5) and 0.2 μ g of purified turkey gizzard calponin (lane 7) were labelled with the polyclonal antibody to gizzard calponin. Specificity for calponin was probed by using the saturated polyclonal antibody to gizzard calponin on a parallel aliquot of glial cells (lane 6).

a comparison with a homologous rat smooth-muscle protein, an aliquot of rat trachea extract was analysed. It showed the presence of a major immunoreactive 34 kDa protein and a less-reactive 70–71 kDa protein doublet (Figure 2, lane 5).

Detection of calponin isoforms in pig brain

In all subsequent experiments, in order to limit the formation of disulphide bonds through oxidation, proteins were incubated for 1 h at 37 °C with 0.2% SDS/1 M β -mercaptoethanol/30 mM

Tris/HCl, pH 8.8. Aliquots were mixed with standard Laemmli sample buffer supplemented with 200 mM DTT, boiled for 2 min at 100 °C before electrophoresis.

We have investigated whether a calponin-like protein could be detected in pig brain. When proteins fractionated with 45% $(\text{NH}_4)_2\text{SO}_4$ (25.8 g/100 ml of extract) were analysed by Western blot, a major 36–37 kDa and a doublet of 72–74 kDa proteins were immunostained. This pattern resembles that described for rat brain, suggesting that pig brain contains a calponin-like protein, although its molecular mass seems slightly higher (Figure 3, lane 4). The rat and pig brain calponin-immunoreactive proteins are different from any degradation products of adducin (97–105 kDa), another calcium-dependent calmodulin-binding protein first discovered in erythrocytes [24] and further characterized in brain [25]. This protein, in contrast with caldesmon [14], does not cross-react with the polyclonal antibody to calponin, as illustrated on Figure 3(c). Subunits of tropomyosin from rat or pig brains show molecular masses of 32–38 kDa [15,26], values in the range of those of the immunoreactive calponin-like proteins that we have characterized. However, a negative result has been observed with purified pig brain tropomyosin, eliminating the possibility that this protein might have a common epitope with calponin which could be recognized by the antibody to gizzard calponin (Figures 3a and 3b). We have also checked that affinity-purified polyclonal antibody to pig platelet caldesmon did not label the 35–36 kDa calponin-like protein (results not shown).

Calponin-immunoreactive protein in rat cerebellar cultured cells

To eliminate the possibility that this calponin-immunoreactive protein might be generated by contamination with brain capillary-vessel cells and to assess the presence of a calponin-like protein in the brain, we have looked for this protein in neurons and in glial cells cultured from rat cerebellum. As shown in Figure 4, in both types of cells a major 35–36 kDa and a less-reactive 70–72 kDa protein were immunostained, the amount of the latter varying from assay to assay. No labelling was observed with the polyclonal antibody saturated with gizzard calponin.

Figure 5 Distribution of calponin in cultured cerebellar cells

Neurons were labelled with monoclonal antibodies to actin (1:100) (a) or to MAP2 (1:500) (c) developed with Rhodamine-conjugated anti-mouse IgG (1:100; Pierce). The same cells were further labelled with polyclonal antibody to gizzard calponin (5 μ g/ml) (b and d) further developed with biotinylated anti-rabbit IgG (1:100; Amersham) followed by avidin conjugated with fluorescein (1:500; Pierce). Glial cells were labelled with polyclonal antibody to GFAP (1:200) developed with Rhodamine-conjugated anti-rabbit IgG (1:100; Chemicon) (e). The same cells were further labelled with monoclonal antibody to gizzard calponin (1:100) revealed with biotinylated anti-mouse IgG (1:400; Dako) followed by fluorescein-conjugated avidin (1:500; Pierce) (f). Calponin distributes within the cell body and in dendritic-like processes in the granular cells. It accumulates in the nuclei of glial cells (arrows) and along cytoplasmic filamentous structures (f). The bar represents 200 μ m in (a) and (b), and 100 μ m in (c-f).

Double-immunofluorescence cytochemical studies

The presence of calponin in brain cells has also been emphasized by double-immunofluorescence cytochemical studies. For the purpose of comparison, neurons were first labelled with antibody to actin (Figure 5a) or to microtubule-associated protein 2 (MAP2; Figure 5c) and glial cells with antibody to GFAP (Figure 5e) and then with antibody to gizzard calponin. Both types of cerebellar cells were calponin-immunoreactive. As shown in Figures 5(b) and 5(d), calponin distributes in the cell body and

along the dendritic processes of the granular neurons in a punctate pattern and on fibrillar structures in glial cells (Figure 5f). It is also noteworthy that antibody to calponin strongly stains the nuclei of glial cells (Figure 5f).

Comparison of the rat brain isoform of calponin with its gizzard counterpart

Finally, analysis of proteins from an aliquot of rat hippocampus by two-dimensional isoelectric focusing/PAGE, blotting and

Figure 6 Two-dimensional isoelectric focusing/PAGE and immunoblots of total homogenate of rat hippocampus

Hippocampus was directly homogenized in 9.5 M urea/2% Nonidet P40/2% Ampholines (Bio-Rad; pI 3–10)/5% β -mercaptoethanol. (a) Silver-stained gel; (b) corresponding blot of 35 μ g of proteins labelled with polyclonal antibody to gizzard calponin and revealed by the ECL technique developed with autoradiography using Fuji X-Ray film. The same blot was further labelled with the polyclonal antibody to actin and stained with peroxidase and 3,3'-diaminobenzidine. The sheet of Immobilon and the X-ray film were superposed (c). Note the acidic pI of the rat brain isoform of the calponin-like protein, which is very close to that of actin. For the purpose of comparison, 0.2 μ g of purified gizzard calponin was submitted to the same two-dimensional isoelectric focusing/PAGE and electroblotting on to Immobilon. The position of the protein as revealed by the ECL technique after antibody treatment is indicated in (d). Open star in (c), actin; arrowheads in (a)–(c), rat brain calponin; arrow in (d), gizzard calponin.

labelling with antibody to gizzard calponin, revealed that the immunoreactive protein displays a pI close to that of actin (5.2–5.4), while gizzard calponin is a basic protein (9.9) (Figure 6). We therefore infer that the calponin isoform identified in the present study corresponds to the acidic isoform of the protein present in rat brain and expressed by the mRNA analysed by Applegate et al. [10].

Conclusion

In the present study we have demonstrated, for the first time, the expression of a calponin-like protein in rat and pig brains. Our work provides a substantial body of evidence to support the notion that the lack of calponin in chicken and bovine brains [2,4] should not be regarded as a specific feature of brain. From all our data we demonstrate that the native calponin-like protein in rat brain has a molecular mass of 35–36 kDa, while its counterpart from pig brain has a molecular mass of 36–37 kDa. Its localization in different parts of the rat central nervous system compared with those of caldesmon and actin is under investigation. Purification of the protein is now necessary in order to study its specific biological properties and its putative involvement in the functions of the brain.

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