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Creatine Kinase Isoenzymes in Chicken Cerebellum: Specific Localization of Brain-type Creatine Kinase in Bergmann Glial Cells and Muscle-type Creatine Kinase in Purkinje Neurons

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Abstract

Creatine kinase isoenzymes were localized in the chicken cerebellum by the use of isoenzyme-specific anti-chicken creatine kinase antibodies. Brain-type creatine kinase was found in high amounts in the molecular layer, particularly in Bergmann glial cells but also in other cells of the cerebellar cortex, e.g. in astrocytes and in the glomerular structures, as well as in cells of the deeper nuclei. A mitochondrial creatine kinase isoform was primarily localized to the glomerular structures in the granule cell layer and was also identified in Purkinje neurons. Surprisingly, a small amount of the muscle-type creatine kinase isoform was identified in cerebellar extracts by immunoprecipitation, immunoblotting and native enzyme electrophoresis, and was shown to be localized exclusively in Purkinje neurons. Cell type-specific expression of brain- and muscle-type creatine kinase in Bergmann glial cells and Purkinje neurons, respectively, may serve to adapt cellular ATP regeneration to the different energy requirements in these specialized cell types. The presence of brain-type creatine kinase in Bergmann glial cells and astrocytes is discussed within the context of the energy requirements for ion homeostasis (K^+ resorption), as well as for metabolite and neurotransmitter trafficking. In addition, the presence of muscle-type creatine kinase in Purkinje neurons, which also express other muscle-specific proteins, is discussed with respect to the unique calcium metabolism of these neurons and their role in cerebellar motor learning.

Introduction

Creatine kinase (CK; ATP: creatine *N*-phospho-transferase, EC 2.7.3.2) catalyses the reversible transfer of the phosphoryl group from phosphocreatine to ADP, to regenerate ATP. The enzyme has been shown to play an important role in the energy metabolism of tissues with high and fluctuating energy demand (for review see Wallimann *et al.*, 1992). In higher eukaryotes, CK exists in highly conserved isoforms. Two cytosolic forms, the sarcomeric muscle-specific M-CK and the more ubiquitous brain-type CK (B-CK), and two mitochondrial isoforms (Mi_a- and Mi_b-CK) have been identified (for review see Wyss *et al.*, 1992).

Although the relative distribution of CK in different areas or cell types of the brain has been investigated in numerous studies, there is still no consistent and complete overview of the localization of CK isoenzymes in the brain. Regional variations in CK activity with comparatively high levels in the cerebellum were reported in studies using native isoenzyme electrophoresis (Chandler *et al.*, 1988) or enzymatic CK activity measurements of either tissue extracts (Maker *et al.*, 1973) or cultured brain cells (Manos *et al.*, 1991). In particular, the molecular layer of the cerebellar cortex contains high levels of CK activity (Maker *et al.*, 1973; Kahn, 1976), consistent with recent ^{31}P nuclear magnetic resonance findings which indicate that grey matter shows a higher flux through the CK reaction and higher phosphocreatine concentrations as compared with white matter (Cadoux-Hudson *et al.*, 1989). In contrast, high levels of either CK activity or the corresponding mRNA were shown in cultured oligodendrocytes (Manos *et al.*, 1991; Molloy *et al.*, 1992), typical glial cells of the white matter.

Conflicting results have also been obtained using histochemical and immunohistochemical techniques; one report indicated that B-CK was present exclusively in astrocytes of the human brain (Thompson *et al.*, 1980), whereas other research groups localized B-CK to both astrocytes and neurons, with a prominent CK content in large neurons of the rat, human, gerbil and mouse brain (Kahn, 1976; Yoshimine *et al.*, 1983, 1984; Ikeda and Tomonaga, 1987). However, in none of the immunohistochemical studies was a complete set of antibodies against

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all CK isoenzymes of the respective species available; often, heterologous antibodies, made against CK isozymes of different animal species, were used for the localization studies.

In addition to these uncertainties in the cellular distribution of CK isozymes, the number and the relative quantities of the different isoenzymes present in the brain are also under debate. B-CK isoenzymes are generally believed to be the main or even exclusive cytosolic CK isoform in the brain, and high quantities of B-CK were purified from brain tissue of the rabbit, human and chicken (Armstrong *et al.*, 1977; McBride and Rodgerson, 1984; Quest *et al.*, 1989). However, in several early studies, using only native starch gel or cellulose polyacetate electrophoresis to analyse brain extracts, so-called 'cathodic subforms' of CK, interpreted to represent M-CK, were reported in vertebrate brain (Lindsey and Diamond, 1978; Ramirez and Licea, 1982). Since different oligomeric forms of mitochondrial creatine kinase (Mi-CK), which were not known to exist at that time, could also give rise to cathodic subforms that display, depending on the species, very similar mobilities to M-CK, some of these reports should be judged with caution. On the other hand, M-CK could indeed be demonstrated recently in postmortem human brain extracts by biochemical isolation and protein sequencing (Hamburg *et al.*, 1990).

In the chicken, four CK isoenzymes are known. Chicken B-CK is the cytosolic isoform expressed in most chicken tissues (Quest *et al.*, 1989), and was purified from brain, retina, gizzard and heart tissues (Quest *et al.*, 1989). Chicken M-CK is specifically expressed in chicken skeletal muscle. Some of the M-CK in sarcomeric muscle was shown to be associated with the M-line and with the sarcoplasmic reticulum. At these sites M-CK is functionally coupled to the myofibrillar myosin ATPase (Wallimann *et al.*, 1984) and the Ca^{2+} pump (Rossi *et al.*, 1990), respectively, to regenerate ATP and to maintain high local ATP/ADP ratios. Two mitochondrial CK isoforms exist in chickens, similar to the sarcomeric and ubiquitous Mi-CK isoforms found in humans (reviewed in Wyss *et al.*, 1992). Mi_b-CK is expressed in heart and skeletal muscle, and Mi_a-CK is found in brain and other tissues (Hossle *et al.*, 1988; Wyss *et al.*, 1990, 1992). All chicken CK isoenzymes have been purified to apparent homogeneity (Caravatti *et al.*, 1979; Wallimann *et al.*, 1984; Schlegel *et al.*, 1988; Quest *et al.*, 1989; Wyss *et al.*, 1990), and specific antibodies against B-, M-, Mi_a- and Mi_b-CK have been generated and characterized in this laboratory. We were therefore in a position to investigate the cellular distribution and localization of all chicken CK isoenzymes within the chicken brain. Here we report an analysis of the CK isoenzyme distribution in the cerebellum, which was chosen due to its geometrically simple, laminated structure and because it contains a limited number of cell types, all of which have been well described (Palay and Chan-Palay, 1974).
Parts of this work have been presented in an abstract (Hemmer *et al.*, 1993a).

Materials and methods

Purification of enzymes and preparation of antibodies

The chicken isoenzymes Mi_a-CK (Wyss *et al.*, 1990), B-CK (Quest *et al.*, 1989) and M-CK (Caravatti *et al.*, 1979; Wallimann *et al.*, 1984) were prepared as described. A newly generated rabbit anti-B-CK serum was prepared as described (Perriard *et al.*, 1978), using type-I B-CK eluted from a Mono-Q column (Quest *et al.*, 1989).

Immunofluorescence labelling

Chicken cerebella or whole brains from 4-week-old chickens were fixed by immersion in either a mixture of ethanol/acetic acid (19/1; fixative A), methanol/acetic acid (9/1; fixative B) or Carnoy's solution (chloroform/acetic acid/ethanol, 30/10/60; fixative C) for 1 h at 4°C, bisected sagittally, fixed for an additional 6 h in the same fixative and transferred to 100% ethanol. Then the tissues were immersed in chloroform and embedded in paraffin by standard techniques. Sections of 5 μm thickness were cut, deparaffinized with xylene, rehydrated by a reversed graded series of ethanol and washed with Tris-buffered saline (TBS: 150 mM NaCl, 50 mM Tris−HCl, pH 7.4). Non-specific binding sites were blocked with TBG [1% bovine serum albumin (BSA), 0.2% gelatine in TBS] for 30 min and incubated with primary antibodies, generally at dilutions between 1:50 and 1:200 in TBG, for 2 h in a moist chamber, followed by washing for 30 min in three changes of TBS. Rhodamine-conjugated goat anti-rabbit IgG (Pierce, 1:500 dilution) or fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Nordic, 1:100 dilution) were both diluted in TBG and incubated on the specimen for 1 h. After three washes with TBS, specimens were mounted in buffered polyvinyl alcohol medium in the presence of *p*-phenylene diamine (1 mg/ml) as anti-fading reagent.

The following primary antibodies against chicken B-, M-, Mi_a- and Mi_b-CK isoenzymes, previously generated and characterized in this laboratory, were used for immunolocalization: rabbit anti-Mi_a-CK serum (Wyss *et al.*, 1990); rabbit anti-Mi_b-CK serum (Wallimann *et al.*, 1986; Schlegel *et al.*, 1988), rabbit anti-M-CK antiserum (Caravatti *et al.*, 1979; Wallimann *et al.*, 1984; Rossi *et al.*, 1990); rabbit anti-M-CK IgG cross-absorbed on B-CK (Perriard *et al.*, 1978); monoclonal mouse anti-M-CK antibody (Cerny, 1984); furthermore, the polyclonal rabbit anti-M-CK serum was affinity-purified on an M-CK Sepharose affinity column (Schlegel *et al.*, 1988). In addition, a newly generated rabbit anti-B-CK serum, giving identical results to the previously characterized (Wallimann *et al.*, 1986; Hemmer et al., 1993b) rabbit anti-B-CK sera and IgG (not shown), was used. In control stainings, preimmune sera were used at the same dilutions as the corresponding immune sera.

In the case of anti-B-/anti-M-CK double immunofluorescence labelling, the tissue sections were incubated with goat anti-B-CK antibody (Quest *et al.*, 1990), diluted at 1:20, together with rabbit anti-M-CK serum (Caravatti *et al.*, 1979; Wallimann *et al.*, 1984; Rossi *et al.*, 1990), followed by staining with corresponding secondary antibodies, donkey anti-goat IgG and swine anti-rabbit IgG (both from Nordic). Anti-Mi_a-CK/anti-GFAP double labellings (not shown) and anti-B-CK/anti-GFAP double stainings were performed using monoclonal mouse anti-GFAP antibody (Boehringer, 1:20 dilution) together with rabbit anti-Mi_a-CK or rabbit anti-B-CK antibodies (described earlier), in combination with goat anti-mouse IgG coupled to rhodamine (Calbiochem) and with FITC-conjugated pig anti-rabbit IgG (Nordic) as secondary antibodies, diluted at 1:200.

Confocal laser microscopy

The samples for confocal microscopy were prepared identically to those for conventional fluorescence microscopy. The confocal system consisted of a Zeiss Axioplan fluorescence microscope, a Bio-Rad MRC-600 confocal scanner and a Silicon Graphics workstation computer. The scanner allowed two fluorochromes to be recorded at the same time. The fluorochromes were excited at 488 nm (FITC) and 568 nm (Texas red) using an argon−krypton mixed gas laser. A background value corresponding to the first relative minimum in the grey level histogram was subtracted from all data prior to any reconstruction (Pawley, 1990). Data are presented in false colours. The axial (z-) dimension of the confocal image was ~0.7 μm.

Electrophoretic analysis and immunoblotting

Tissue extracts were analysed by 10% polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulphate (SDS). Blotting was

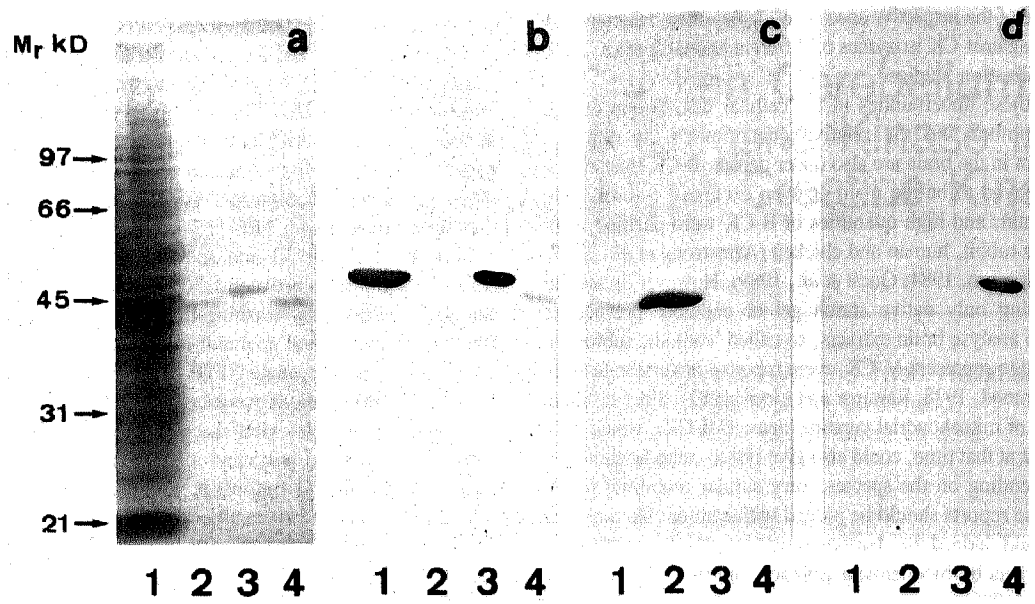

FIG. 1. Isoenzyme specificity of the anti-CK antibodies used for immunofluorescence labelling and immunoblot characterization of chicken cerebellar extracts. Cerebellar extracts (80 μg, lane 1) as well as purified Mi_a-, B- and M-CK (0.5 μg each, lanes 2, 3 and 4, respectively) were analysed by SDS–PAGE, subsequently blotted onto nitrocellulose and stained with Ponceau red S (panel a). Panels b, c and d represent parallel blots, destained with PBS followed by antibody labelling with anti-B-, anti-Mi_a- and anti-M-CK antibodies, respectively. CK antibodies were highly isoenzyme-specific (compare lanes 2–4 in panels b, c and d), and each recognized a single protein band of slightly different M_r in the range 42–45 kDa in total cerebellar extracts. The apparent molecular masses of marker proteins are indicated at the left in kDa.

performed with semidry blotters (JKA-Biotech, Denmark), using the continuous buffer system described by the manufacturer. Transfer efficiency was controlled by staining of protein with Ponceau red S, prior to blocking non-specific binding sites on the nitrocellulose with blocking buffer, containing 1% BSA in phosphate-buffered saline (PBS: 137 mM NaCl, 2.6 mM KCl, 6.4 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4). Specific labelling of B-, Mi_a- and M-CK on the blots was achieved with antiserum solutions diluted 1:800 in blocking buffer, followed by three wash cycles with PBS and incubation with peroxidase-conjugated goat anti-rabbit IgG (Nordic), diluted at 1:3000. The anti-B-, Mi_a- and M-CK antibodies were the same as those used for immunolocalization. After three wash cycles with PBS, blots were developed, using 4-chloro-1-naphthol and H_2O_2 as substrate. Two-dimensional gel analysis was done as described (Hemmer *et al.*, 1993b). Immunoblotting of proteins separated by two-dimensional gels was performed as described above, again using polyclonal anti-M-CK serum at a dilution of 1:1000.

Immunoprecipitation

Cerebellar extracts were diluted with buffer A (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 20 mM Tris, pH 7.4) to a final protein concentration of 2 mg/ml and immunoprecipitated as described (Hemmer *et al.*, 1993b). Briefly, extracts were preincubated with protein A-coated Sepharose beads (Sigma) for 2 h on ice and centrifuged for 2 min at 1000 *g*. Anti-chicken M-CK antiserum (5 μl) was added to the supernatant and incubated for 1 h on ice, followed by precipitation of the immune complex with protein A-coated Sepharose beads. After extensive washing with buffer A the immunoprecipitates were analysed by two-dimensional gel electrophoresis and immunoblotting. The anti-M-CK serum was the same as that used for immunoblotting and immunolocalization.

Preparation of tissue extracts and cellulose polyacetate electrophoresis

Chicken cerebellar extracts (1 volume) were homogenized in 2.6 volumes of ice-cold hypotonic buffer B (0.5 mM EGTA, 1 mM PMSF, 2 mM β-mercaptoethanol), using a glass–Teflon homogenizer. The homogenate was kept for 15 min on ice before buffer C (buffer B supplemented with 1 M NaH_2PO_4, pH 8.4) was added to give a final concentration of 0.1 M phosphate and a tissue to volume ratio of 1 : 4. Triton X-100 was added to 0.05%, thus providing complete solubilization of CK isoenzymes. Samples were centrifuged for 15 min at 100 000 *g* in an air fuge (Beckmann) at 4°C. Native cellulose polyacetate gel electrophoresis (CPAGE) was performed at pH 8.45, essentially as described elsewhere (Wyss *et al.*, 1990), except that the separated isoenzymes were stained in the presence of 0.3 mM P^1,P^5-di(adenosine-5′)-pentaphosphate (Ap5A); this concentration of Ap5A was sufficient to inhibit even high activities of adenylate kinase (Fig. 8, lane 1).

Results

Isoenzyme specificity of the antibodies used for the localization of CK isoenzymes within chicken cerebellum

We have made use of a set of isoenzyme-specific antibodies for the unambiguous localization of all known chicken CK isoenzymes in adult chicken cerebellum. All antibodies had been raised against highly purified homogeneous chicken isoenzymes (B-, Mi_a- and M-CK) and were shown by Western blotting to be highly isoenzyme-specific (Fig. 1). Blots of SDS gels on which Mi_a-, B- and M-CK were run in parallel were probed with antibodies specific for each of these isoenzymes. No isoenzyme cross-reactivity was found using anti-M- and Mi_a-CK antibodies (Fig. 1, lanes 2–4 of panels d and c, respectively) and only a very slight cross-reactivity of the anti-B-CK antibody to M-CK was detectable (Fig. 1, compare lanes 3 and 4 in panel b).

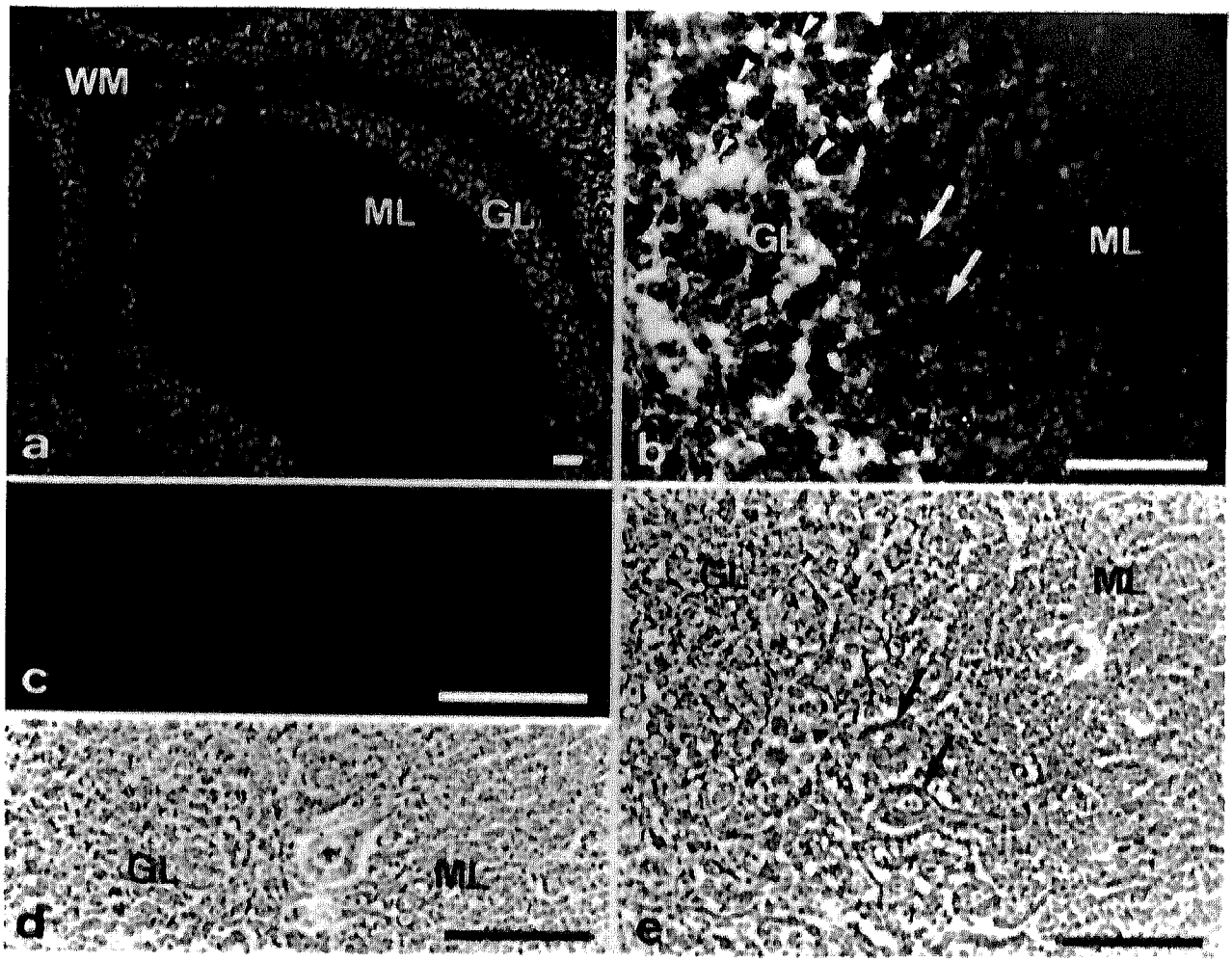

FIG. 2. Localization of Mi_a-CK in chicken cerebellum by immunofluorescence microscopy. Paraffin sections of chicken brain, fixed with fixative A (b, e) or B (a, c, d), were labelled with rabbit anti-Mi_a-CK antibody (a, b) or preimmune serum (c) at the same dilutions (1:300), followed by rhodamine-coupled IgG. Panels d and e are phase-contrast pictures corresponding to panels c and b, respectively. Note the strong staining of the glomeruli, marked by arrowheads in panel b, located in the granule cell layer (GL). Low staining levels were found in the molecular layer (ML) and in the white matter (WM). Purkinje cell bodies are indicated by arrows in panels b and e. Bars represent 50 μm in all panels.

Localization of mitochondrial CK isoforms

Mi_b-CK is expressed in chicken heart and skeletal muscle, whereas Mi_a-CK is expressed in most other chicken tissues, e.g. brain (Hossle *et al.*, 1988), from which it was originally purified (Wyss *et al.*, 1990). In accordance with this, immunofluorescence studies on paraffin sections of adult chicken cerebellum with polyclonal anti-Mi_a-CK antibody revealed rather strong specific labelling within the granule cell layer (Fig. 2a). The staining pattern within this layer strongly indicated that glomerular structures (Palay and Chan-Palay, 1974) were labelled (Fig. 2b, arrowheads). Perinuclear staining of neurons or glial cells within the granule cell layer was less intense than the staining of the glomerular structures. Purkinje neurons, located at the interface between the molecular and granule cell layers (long arrows in Fig. 2), also express some Mi-CK, as indicated by the weak spotted staining that is typical of the mitochondrial staining (Hawkes *et al.*, 1982) which is seen with polyclonal anti-Mi_a-CK antibody. However, very recent results using anti-peptide antibodies against Mi_b-CK, that is against the sarcomeric Mi-CK isoform, suggest that Purkinje neurons preferentially express this latter isoform (Kaldis, personal communication), indicating that the slight staining of Purkinje neurons with anti-Mi_a-CK antibody may be due to cross-reactivity of the anti-Mi_a-CK antibody with the highly homologous Mi_b-CK isoenzyme. Other cell bodies and processes in the molecular layer were stained only very slightly. In some control labellings using preimmune sera, staining of cerebellar white matter occurred; thus, occasional staining of this area by anti-Mi_a-CK antibody was judged to be non-specific.

Localization of B-CK

In the adult chicken cerebellum, anti-B-CK antibodies labelled structures throughout the cerebellar cortex, the most prominent immunostaining being observed in the molecular layer (Fig. 3a). Labelling in the granule cell layer was less prominent. Higher magnifications revealed that, while Purkinje neurons themselves and their primary dendrites remained essentially unstained (Fig. 3d, large arrow), intense B-CK labelling was seen in those cells (indicated by arrowheads in Fig. 3d) which surround Purkinje neurons. The outline of these cells, which send straight radial processes through the entire molecular layer to the membrana limitans (arrowheads in Fig. 3a), can easily be followed by the anti-B-CK immunofluorescence signal. Based on their general morphology and location, these cells were identified as Golgi epithelial cells (Rakic, 1971).

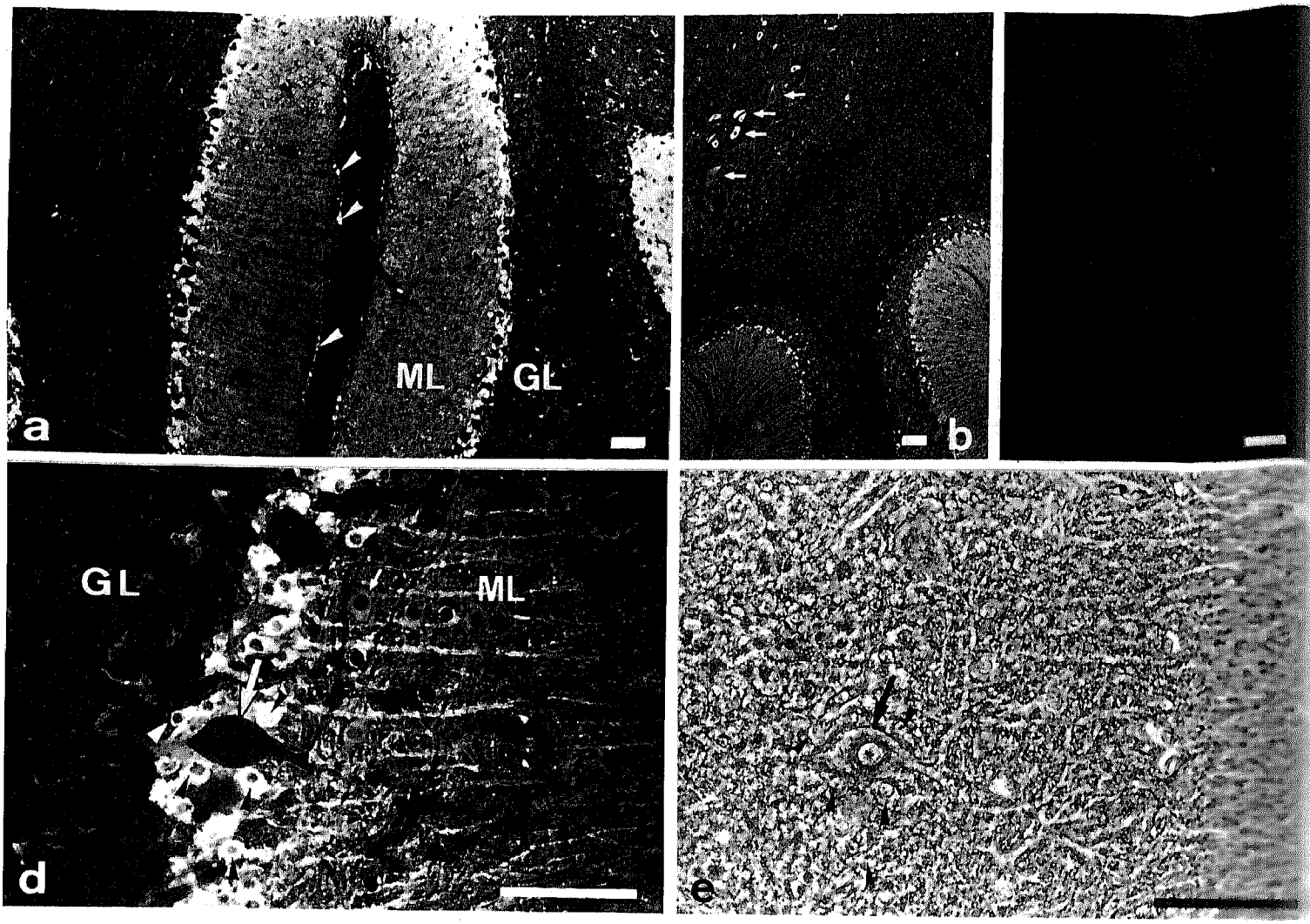

FIG. 3. Localization of B-CK in chicken cerebellum by immunofluorescence microscopy. Paraffin sections of chicken brain, fixed with fixative A (a, c, d, e) or B (b), were labelled with rabbit anti-B-CK antibody (a, b and d) or preimmune serum (c), followed by secondary antibodies coupled to FITC (a, d) or rhodamine (b, c). The phase-contrast picture corresponding to panel d is shown in panel e. Note the intense staining of the molecular layer (ML) compared with the granule cell layer (GL); in particular, cell bodies of BGC (arrowheads in panel d) and their processes, spanning through the molecular layer and finally building the membrana limitans (arrowheads in panel a) are strongly stained for B-CK. Purkinje neurons, indicated by large arrows in panels d and e, remained unstained, whereas other neurons within the molecular layer (small arrows in panel d) and in the deeper nuclei (arrows in b) also showed significant anti-B-CK immunoreactivity. Controls did not show any significant labelling (c). Bars represent 50 μm in all panels.

The typical straight processes of these cells in the molecular layer are often referred to as Bergmann fibres and the cells themselves as Bergmann glial cells (BGC). Since BGC processes span the entire molecular layer and build up the membrana limitans gliae, most of the heavily stained structures in the molecular layer can be attributed to BGC.

Other cell types in the molecular layer, e.g. basket or stellate cells (Fig. 3d, small arrow), were also stained by anti-B-CK antibody, albeit to a lesser extent than BGC. Additionally, structures around the granule cells that were identified above as glomerular structures and shown to contain Mi_a-CK (Fig. 2) were also slightly stained by the anti-B-CK antibody, whereas the granule cell bodies themselves remained rather unstained. In the deeper nuclei of the cerebellum some large cells were intensely stained with anti-B-CK antibody (arrows in Fig. 3b). As judged from their size and shape, these cells were assumed to be neurons. In control stainings using preimmune sera or control IgG, except for some non-specific staining of blood vessels no significant labelling in any of the cerebellar layers was observed (Fig. 3c). Astrocytes in the granule cell layer, which were identified by staining with an antibody against GFAP, also contained significant anti-B-CK immunoreactivity, as determined by double immunofluorescence staining (Fig. 4). Interestingly, and in accordance with the findings of Debus *et al.* (1983), in the chicken cerebellum only the astrocytes of the granular layer, but not BGC, were stained with anti-GFAP antibody; this is in contrast to other vertebrate species, such as the rat, in which BGC are heavily labelled using the same anti-GFAP antibodies (Debus *et al.*, 1983; not shown).

Localization of 'muscle-specific' M-CK

In situ staining of paraffin sections from chicken cerebellum by isoenzyme-specific anti-M-CK serum revealed an intriguing labelling pattern. Staining was found specifically in cerebellar Purkinje neurons (Fig. 5a). Higher magnification revealed significant anti-M-CK

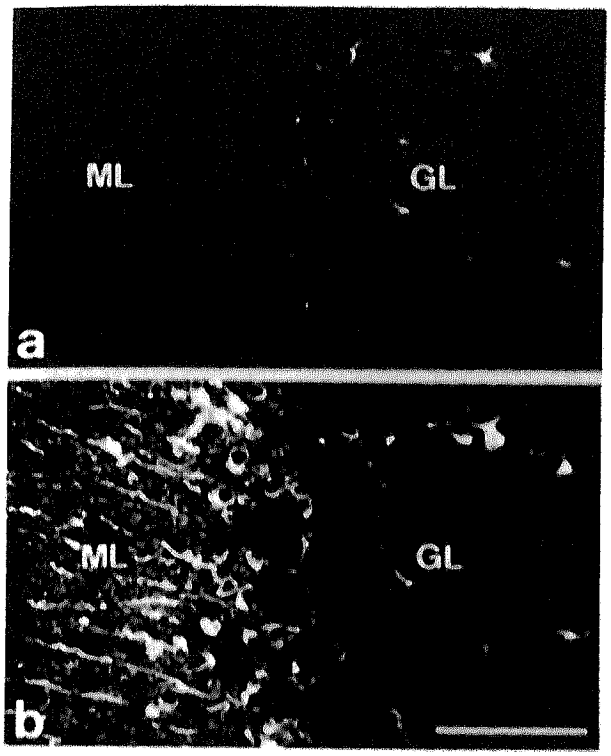

FIG. 4. Anti-GFAP/anti-B-CK double immunofluorescence labelling. A section of chicken cerebellum, fixed with fixative B, was double-labelled with anti-GFAP (a) and anti-B-CK antibodies (b). The same area is illustrated in panels a and b. Anti-B-CK staining was similar to that in Figure 3. Astrocytes in the granule cell layer (GL) were identified by the antibody against the astrocyte marker GFAP. The staining pattern within the granule cell layer was very similar for the two antibodies (compare panels a and b), indicating that most of the GFAP-positive astrocytes in the granule cell layer also contain B-CK. Note that chicken BGC do not stain with anti-GFAP antibody (Debus *et al.*, 1983; see also Results). Bar represents 50 μm in both panels.

immunoreactivity in Purkinje neuron perikarya as well as in their primary dendrites and dendritic arborizations (Fig. 5d). The labelling of the dendrites was stronger than that of the Purkinje cell bodies, with most intense staining in the proximal parts of the Purkinje cell dendrites and decreasing staining intensity towards the finer arborizations of the dendritic tree. The specific staining of Purkinje neurons by anti-M-CK antibody was further corroborated by the use of several other anti-M-CK antibodies, including anti-M-CK IgG that was cross-absorbed on a B-CK column, monoclonal anti-M-CK antibody, and affinity-purified anti-M-CK IgG (not shown).

Cell type-specific expression of B- and M-CK in the cerebellum

Whereas M-CK was exclusively localized within the Purkinje neurons (Fig. 5), these cells were essentially unlabelled when brain sections were stained with anti-B-CK antibody. In contrast, anti-B-CK antibody labelled the neighbouring BGC most intensely (Fig. 3). This cell type-specific localization of the two cytosolic isoforms B- and M-CK could be directly illustrated by double immunofluorescence labelling with anti-B- and M-CK antibodies, analysed by confocal microscopy, followed by digitized image processing (Fig. 6). The M-CK-containing Purkinje neurons are visible in red between B-CK-containing BGC, illustrated in green. The proximal parts of Purkinje cell dendrites contain the most anti-M-CK immunoreactivity and thus display the highest red intensities. As in Figure 5, the distribution of M-CK within the Purkinje neurons and their dendrites was non-uniform.

Identification of M-CK in cerebellar extracts by immunoprecipitation, two-dimensional gel and native isoenzyme electrophoresis

The localization studies showed that Purkinje neurons in the chicken cerebellum contain a CK that cross-reacts with a number of anti-chicken M-CK antibodies. To demonstrate that this CK was truly M-CK, brain extracts were examined by biochemical means. Western analysis of cerebellar extracts showed that both anti-B-CK and anti-M_4 CK antibodies stained single bands in the molecular weight range expected for these CK isoforms (Fig. 1, lane 1, panels b and c respectively). However, incubation with anti-M-CK antibodies did not lead to significant staining (Fig. 1d, lane 1); this might reflect the fact that probably only a single cell type expresses M-CK within this tissue and indicates that the overall amount of M-CK is relatively low. Therefore, we decided to investigate chicken cerebellar extracts for the presence of M-CK by two-dimensional gel analysis. Based on their different, slightly acidic isoelectric points, chicken M- and B-CK can be separated on two-dimensional gels with a pH range from 5 to 8 (Schäfer and Perriard, 1988). M_4-CK, which migrates with a similar mobility to M-CK in SDS gels, has a very basic pI and consequently does not enter isoelectric focusing gels with this pH interval.

Two-dimensional gel analysis of chicken cerebellar extracts, followed by silver staining, revealed intensely labelled protein spots at the positions where the two well-known B-CK subunit forms (Schäfer and Perriard, 1988; Quest *et al.*, 1989; Hemmer *et al.*, 1993b) are located (Fig. 7a, arrowheads), whereas M-CK was not detectable. However, by immunoprecipitation of cerebellar extracts with anti-M-CK serum, followed by two-dimensional gel analysis and immunoblotting, two protein spots were clearly revealed, both migrated at the expected molecular weight of ~42 kDa (Fig. 7b, arrows) with a more prominent basic spot, a pattern which was described earlier for purified M-CK subforms (Schäfer and Perriard, 1988). The same M-CK spots could also be detected when two-dimensional gels of cerebellar extracts were analysed by immunoblotting using anti-M-CK antibody (Fig. 7c, arrows). Upon prolonged exposure the two B-CK subunits also became apparent (Fig. 7c, arrowheads). When the same blot was subsequently stained with anti-B-CK antibody, only the labelling intensities of the B-CK spots (indicated by arrowheads) increased (Fig. 7d), while the presumed M-CK spots remained unchanged. In addition, when purified chicken muscle M-CK was mixed with chicken cerebellar extract and the mixture was analysed by two-dimensional gel electrophoresis and anti-M-CK staining (Fig. 7e), the purified M-CK comigrated exactly with the presumed M-CK spots (Fig. 7b and c). No signals at the corresponding positions were found in control experiments, using preimmune serum (not shown). These experiments clearly proved that the protein spots detected by both immunoprecipitation (Fig. 7b) and two-dimensional Western analysis (Fig. 7c) of cerebellar extracts with anti-M-CK antibody are not related to B- or M_1-CK but are indeed genuine muscle type CK.

As an additional line of evidence that some M-CK is present in cerebellum, we subjected chicken cerebellar extracts to native CK isoenzyme electrophoresis on cellulose polyacetate sheets (CPAGE). B- and M_1-CK were by far the predominant CK isoforms present in cerebellum (Fig. 8). However, a very faint band could also be seen in the lane of the cerebellar extract (Fig. 8, lane 2, asterisk) migrating with the same mobility as dimeric M-CK (MM-CK) from leg muscle (Fig. 8, lane 4). After a cycle of freeze–thawing in order to monomerize CK proteins, the extracts showed a band with the mobility of heterodimeric M-CK/B-CK (MB-CK) (Fig. 8, lane 3), again indicating that some homodimeric MM-CK had been present in the native extracts. In all CPAGE experiments shown, Ap5A was present at a concentration

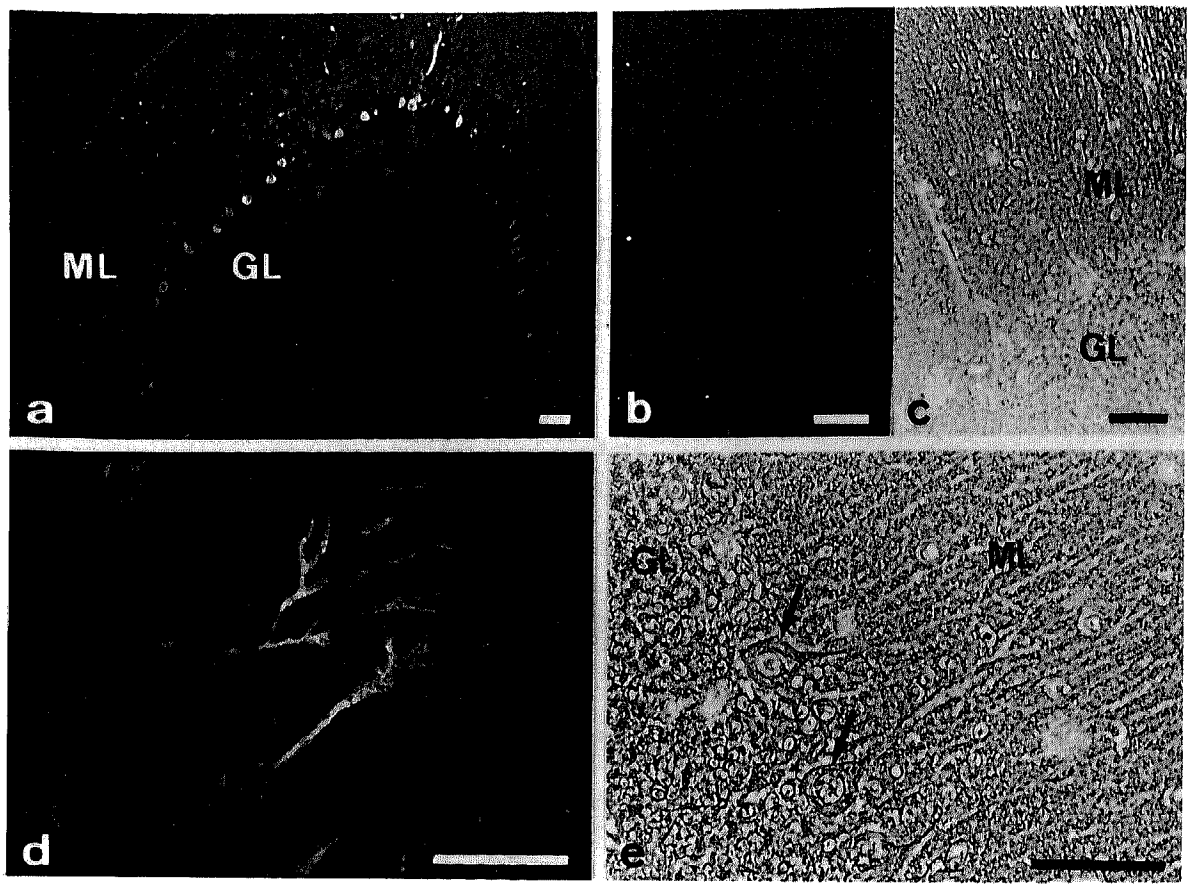

FIG. 5. Localization of 'muscle-type' M-CK in chicken cerebellum. Sections of chicken cerebellum, fixed with fixative B (a, b, c) or C (d, e), labelled with anti-M-CK antibody (a, d) or preimmune serum (b), followed by rhodamine-coupled IgG. Panels c and e are phase-contrast pictures corresponding to panels b and d, respectively. Purkinje neurons, marked by arrows in panel e, were specifically labelled (see panels a and d). Note that staining was most intense in the proximal part of the uniplanar Purkinje cell dendritic tree (d) and that immunoreactive components were non-uniformly distributed. Bars represent 50 μm in all panels.

which was shown to be sufficient to inhibit high activities of adenylate kinase (Fig. 8, lane 1).

Thus, the presence of M-CK in the chicken cerebellum was unambiguously demonstrated, and the very same M-CK isoform was localized exclusively to Purkinje neurons.

Discussion

Isoenzyme-specific localization of creatine kinase isoenzymes in the cerebellum

In this study, the cerebellar localization of chicken CK isoenzymes B-, M- and Mi-CK was determined by conventional immunofluorescence and laser confocal microscopy (Figs 2–6), using different, highly isoenzyme-specific anti-CK antibodies (Fig. 1). Out of several fixation protocols tested, including fixation by 2–6% paraformaldehyde plus 0.1–0.2% glutaraldehyde, the fixatives described here proved to be best suited for achieving maximal preservation of CK antigenicity and for the maintenance of isoenzyme specificity (avoidance of cross-reactivity of the antibodies), as well as for the prevention of significant leakage of soluble CK. However, it has to be pointed out that some cell shrinkage can occasionally be observed in these preparations and that cells which contain only small amounts of CK may not necessarily all be fully stained, due to some limitations of the method applied.

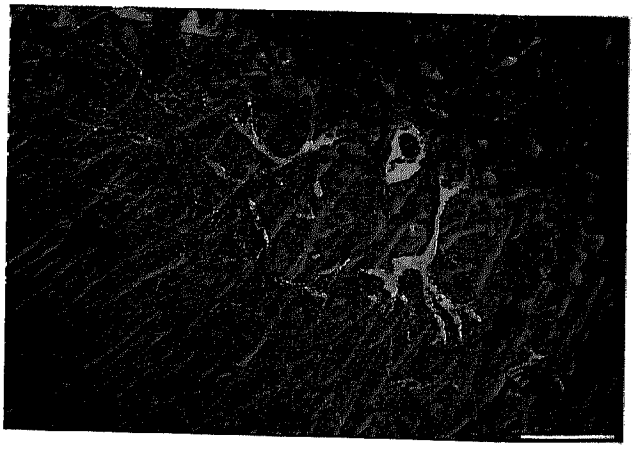

FIG. 6. Cell type-specific localization of B- and M-CK in chicken cerebellum. Confocal laser scanning double immunofluorescence image of M- and B-CK localized in chicken cerebellum, fixed with fixative C. M- and B-CK, visualized in red and green, respectively, are expressed in a complementary and cell type-specific manner. M-CK (red) was exclusively localized in the Purkinje neurons, with prominent staining in the proximal part of the dendritic tree. B-CK (green) was predominantly found in the BGC surrounding Purkinje neurons. Bar represents 50 μm.

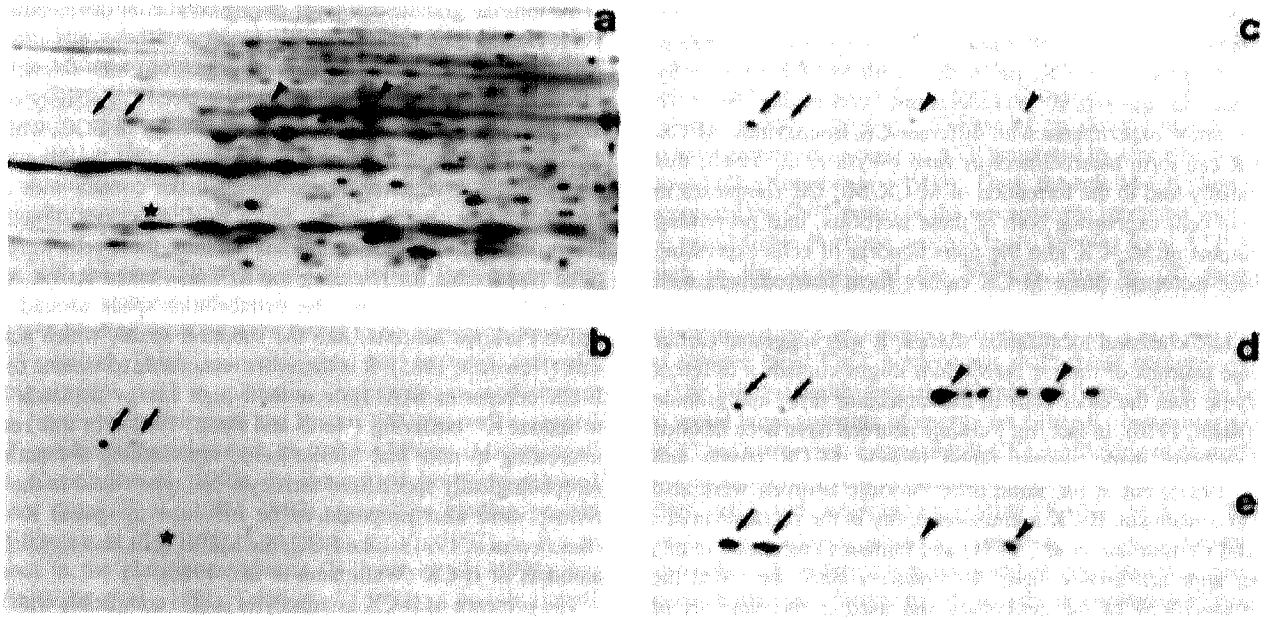

FIG. 7. Two-dimensional gel analysis of anti-M-CK immunoprecipitates of cerebellar extracts and Western analysis of chicken cerebellar proteins separated by two-dimensional gel electrophoresis. Proteins (100 μg) of cerebellar extracts (panels a, c, d and e) or immunoprecipitates of cerebellar extract (panel b) were separated by two-dimensional gel electrophoresis (pH range 8–5 from left to right) and visualized by either silver staining (panel a) or immunoblotting with anti-M-CK antibody (panels b, c, d and e). For both immunoprecipitation and immunoblotting, the same anti-M-CK serum as in Figures 1, 5 and 6 was used. Panel d represents the same blot as that shown in c, but subsequently stained also with anti-B-CK antibody (1:1000 dilution). Panel e represents a parallel gel to panel c, in which cerebellar extract (100 μg protein) was comigrated together with 0.1 μg of purified chicken M-CK, in order to demonstrate the identity of the two protein spots (indicated by arrows), specifically detected in chicken cerebellar extracts by immunoprecipitation (panel b) and immunolabelling (panel c), with genuine, purified M-CK from chicken muscle. The positions of the two M-CK and B-CK spots are indicated by arrows and arrowheads, respectively. Asterisks indicate the position of the most basic spot of a two-dimensional-carbamylate marker protein (carbonic anhydrase, apparent molecular weight 30 kDa, Pharmacia), comigrated as a standard in these gels.

Anti-Mi_a-CK serum labelled structures in the granule cell layer (Fig. 2) likely to represent glomeruli (Palay and Chan-Palay, 1974), which are known to contain large numbers of mitochondria. Anti-B-CK staining was found in all layers of the cerebellar cortex as well as in the deeper nuclei of the cerebellum, indicating that a high proportion of the cerebellar cell types contain B-CK. The labelling was most intense in BGC. The processes of these cells, lying in the vicinity of Purkinje neurons, span radially through the entire molecular layer and finally form the membrana limitans (Rakic, 1971). This morphology is perfectly matched by the intense anti-B-CK staining pattern (Fig. 3). Besides BGC, some other cell types in the molecular layer, such as basket cells (Fig. 3d) and neurons in the deeper nuclei (Fig. 3b) contain B-CK. Additionally, structures in the granule cell layer likely to be glomeruli (Palay and Chan-Palay, 1974) as well as astrocytes in the granule cell layer contained significant anti-B-CK immunoreactivity (Fig. 4), whereas cerebellar white matter appears to contain rather low levels of B-CK. The latter finding is consistent with previous histochemical and ^{31}P nuclear magnetic resonance data (Kahn, 1976; Cadoux-Hudson *et al.*, 1989).

Surprisingly, the 'cytosolic' muscle-type CK isoform, M-CK, was specifically and exclusively located to Purkinje neurons (Figs 5 and 6), which were not stained by anti-B-CK antibody (Fig. 3). Using two-dimensional gel analysis and immunoblotting, it was confirmed that the cerebellum contains M-CK, although this isoenzyme constituted only a very minor part of cerebellar CK activity (Figs 1, 7 and 8), consistent with its presence in only a single cell type. From our data, we estimate that the relative amount of M-CK versus total CK activity in the cerebellum is ~1% or less.

Purkinje neurons stained slightly with our previously characterized anti-Mi_a-CK antibody (Fig. 2). Recently, we have generated new isoenzyme-specific anti-Mi_a-CK and anti-Mi_b-CK antibodies, raised

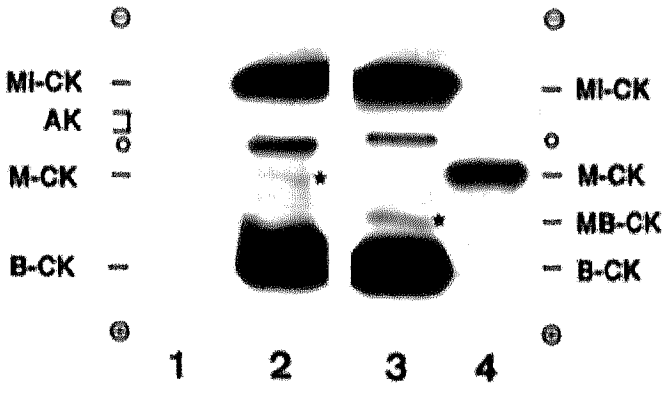

FIG. 8. Cellulose polyacetate electrophoresis of chicken cerebellar extracts. Native cellulose polyacetate electrophoresis of freshly prepared chicken cerebellar extracts at 14 mg protein per ml (lane 2) and after freeze–thawing (lane 3). Lanes 1 and 4 represent chicken adenylate kinase (AK, 0.4 mg/ml) and homodimeric M-CK (0.008 mg/ml), respectively. Adenylate kinase was completely inhibited by 300 μM Ap^5A. The positions of adenylate kinase, Mi_a-CK, homodimeric B- and M-CK, and heterodimeric MB-CK are indicated. Open circles, + and − represent the application points and the anodal and cathodal directions, respectively. Asterisks indicate positions of homodimeric M-CK in native cerebellar extract, as well as MB-CK in the same extract after freeze–thawing, respectively.

against synthetic peptides corresponding to the divergent *N*-terminal amino acid sequence of these isoenzymes; preliminary results using these antibodies indicate that, whereas most other brain cell types express the 'ubiquitous' Mi_a-CK, Purkinje neurons seem to express mainly the 'sarcomeric' Mi_b-CK (Kaldis *et al.* unpublished observation). Thus,

M-CK in Purkinje neurons seems to be coexpressed with the same mitochondrial enzyme as in skeletal muscle. The coexpression in Purkinje neurons of M-CK with Mi_b-CK, rather than with Mi_a-CK, was partly expected due to theoretical considerations concerning *in vitro* heterodimerization experiments with different CK isoenzymes. M-CK and Mi_a-CK can form heterodimers *in vitro* (Wyss *et al.* 1990); this could potentially lead to the formation of M-CK/Mi_a-CK complexes in the cytosol of cells expressing both of these isoforms, thus preventing proper transport of Mi_a-CK into the mitochondria of cells expressing both of these isoforms. Since M-CK cannot form heterodimers with Mi_b-CK (Wyss *et al.* 1990), this potential problem is avoided.

Based on histochemical localization studies, it was suggested earlier that Purkinje neurons of the rat cerebellum might contain a different CK isoenzyme than the other cells of the molecular layer and granule cell layer (Kahn, 1976). In fact, rat Purkinje neurons have been labelled using an antibody made against rabbit muscle M-CK (Ikeda and Tomonaga, 1987), but at the same time Purkinje neurons were also suggested to contain anti-B-CK immunoreactivity in the rat (Kato *et al.*, 1986), gerbil (Yoshimine *et al.*, 1984) and human (Yoshimine *et al.*, 1983). Our approach avoids these discrepancies since we tested the isoenzyme specificity of the antibodies and used a complete set of antibodies for localization in the homologous species.

It has been reported that M-CK constitutes 35% of total CK activity in the postmortem human brain (Hamburg *et al.*, 1990). However, human brain extracts obtained directly at surgery did not contain significant amounts of M-CK (Tsung, 1976), and other human brain extracts contained specific CK activities which were 30-fold higher than those reported from Hamburg and coworkers (Tsung, 1976; McBride and Rodgerson, 1984). Thus, it seems likely that the figure reported by Hamburg *et al.* (1990) was overestimated due to postmortem artefacts.

The finding that chicken B-, M- and Mi-CK are specifically localized in those cells (BGC, Purkinje neurons and glomerular structures) where high and fluctuating energy demands can be inferred is significant. The isoenzyme-specific staining patterns of BGC and Purkinje neurons may indicate that some characteristic properties of the different CK isoenzymes match the distinct energy requirements of these functionally specialized neuronal and glial cell types (see below). Similar differential localizations of specific isoenzymes in cerebellar Purkinje and BGC were reported for protein kinase C (Mochly-Rosen *et al.*, 1987) and enolase (Schmechel *et al.*, 1978) isoenzymes in the rat.

Functional aspects of brain-type CK isoenzymes in the glomerular structures of the granule layer

The granular layer of the cerebellum, especially the glomeruli structures, contains high levels of Mi_a-CK as well as B-CK, as judged from the intensity of antibody staining. These structures, forming intimate synaptic as well as glial–neuron interactions, are known to be rich in mitochondria (Hertz and Peng, 1991). They also display the high energy metabolism needed for restoration of potassium ion gradients, partially broken down during neuronal excitation, as well as for metabolite and neurotransmitter traffic between glial cells and neurons (for review see Hertz and Peng, 1991). Thus, the localization of both B- and Mi_a-CK isoforms within the same structures may be an indication that part of the energy consumed in these giant complexes of mossy fibre, Golgi cell and granule cell synapses, as well as glial cells, might be provided by a 'phosphocreatine circuit', as has been proposed for other excitable cells and tissues (Wallimann *et al.*, 1992).

Functional aspects of brain-type CK isoenzymes in Bergmann glial cells and astrocytes

The BGC is a specialized type of astroglial cell. It provides the migratory pathway of granule cell migration from the external granule cell layer to the internal granule cell layer during cerebellar development (Rakic, 1971; Hatten, 1990). Another main function of these cells is the proposed ATP-dependent spatial buffering of potassium ions (Newman, 1985; Reichenbach, 1991) released during the electrical activity of neurons. This function is also reflected by the morphology of BGC, which envelop the synaptic sites of Purkinje cell dendrites with the exception of the precise sites at which Purkinje spines make contact with parallel or climbing fibres (Rakic, 1971). Since BGC processes directly face the cerebrospinal fluid at the membrana limitans, these cells were suggested to be responsible for releasing the K^+ ions, taken up via ATP-driven Na^+/K^+ ion pumps from the extracellular space around the highly active Purkinje neurons, into the subdural space, which acts as a K^+ sink (Newman, 1985). It is therefore reasonable to assume that the high B-CK content of BGC reflects their high energy demands in relation to spatial K^+ buffering (Hertz and Peng, 1991). In this respect, it is interesting to note that Müller cells, representing a functionally and morphologically specialized astrocyte cell type found in the vertebrate retina, were also proposed to be involved in spatial K^+ buffering (Reichenbach, 1991). Like BGC, the Müller cells also contain significant amounts of B-CK (Wallimann *et al.*, 1986).

The presence of B-CK in astrocytes is also compatible with the energy requirements of these cells, which need energy for metabolic interaction with neurons, e.g. for providing tricarboxylic cycle metabolites to neurons and for exchange and trafficking of neurotransmitters and their precursors between neurons and glial cells (Hertz and Peng, 1991). Since both BGC and astrocytes contain mitochondria and the latter cell type is known to display intense oxidative metabolism (Hertz and Peng, 1991), it is likely that both of these cell types also contain Mi_a-CK, although this cannot be shown unambiguously by our light microscope study, but rather would have to be demonstrated by immunogold labelling. Finally, the high concentration of CK found in oligodendrocytes (Manos *et al.*, 1991) may indicate a function of CK, among other functions, in the energy supply needed for myelinization.

Functional aspects of muscle-type CK isoenzymes in Purkinje neurons

Muscle-type CK is generally thought to be specialized for the energy requirements of skeletal muscle (Schäfer and Perriard, 1988; Wallimann *et al.*, 1992). In chicken, myoid thymus cells were the only cell type in any non-muscle tissue in which M-CK has been located (Perriard *et al.*, 1982). The surprising finding that chicken Purkinje neurons express the muscle-type CK isoform M-CK, and most likely also the muscle-type Mi_b-CK, may reflect the important metabolic features and bioenergetic peculiarities of these specialized brain cells. Purkinje neurons constitute the sole neuronal output of the cerebellar cortex. They receive excitatory input from two neuronal pathways, the climbing and the parallel fibres (reviewed in Ito, 1984). A single Purkinje neuron receives input from a large number of parallel fibres, making synapses at the distal ends of the dendritic tree of the Purkinje neuron. Each activated parallel fibre synapse induces only a small excitatory postsynaptic potential in the Purkinje cell dendrite. In contrast, each Purkinje neuron receives input from only one or a very small number of climbing fibres (Ekerot and Oscarsson, 1981; Ito, 1984), making contact at the more proximal part of the dendritic tree of the Purkinje neuron. A single climbing fibre was estimated to make ~300 synaptic contact sites to a single Purkinje neuron, a unique feature in brain architecture. Thus, the climbing fibres exert powerful excitatory effects on Purkinje neurons. Most importantly, climbing fibre impulses evoke complex Ca^{2+} spikes and prolonged Ca^{2+}-mediated depolarization within Purkinje cell dendrites (Llinás and Sugimori, 1980; Ekerot and Oscarsson, 1981; Knöpfel *et al.*, 1991). The climbing fibre-induced Ca^{2+} influx seems

to play a central role in the mechanisms leading to long-term depression by mediating desensitization of glutamate receptors at the parallel fibre/Purkinje neuron synapse (Ito, 1991). Long-term depression was found to be a characteristic type of synaptic plasticity prevalent in the cerebellar cortex, and was suggested to be basis of cerebellar motor learning (Ito, 1991). The desensitization of glutamate receptors shows interesting similarities with the Ca^{2+}-dependent desensitization of acetylcholine receptors in slow and fast skeletal muscle (Miledi *et al.*, 1981).

Consistent with the powerful effects of Ca^{2+} influx, Purkinje neurons seem to have a very elaborate calcium homeostasis machinery. A whole variety of Ca^{2+}-binding proteins were found to be present in Purkinje neurons in relatively high amounts; this list includes cytosolic Ca^{2+}-binding proteins, such as calbindin, calcineurin, calmodulin and parvalbumin (reviewed in Heizmann and Braun, 1990), as well as several isoforms of membrane proteins such as Ca^{2+}-ATPases (Michelangeli *et al.*, 1991; Campbell *et al.*, 1993), the inositol trisphosphate-activated Ca^{2+} channel (Ross *et al.*, 1989, 1992), ryanodine binding protein isoforms (Ellisman *et al.*, 1990; Kuwajiama *et al.*, 1992), a P-type Ca^{2+} channel in the plasma membrane (Hillman *et al.*, 1991) and calsequestrin (Villa *et al.*, 1991; Takei *et al.*, 1992), a protein typical of the sarcoplasmic reticulum. Antibodies against ryanodine binding protein stained Purkinje cell dendrites preferentially in their proximal parts, reminiscent of the regions where climbing fibre responses in Purkinje neurons are elicited, with a pattern that was indicative of the staining of a complex tubular network (Ellisman *et al.*, 1990). This staining pattern is strikingly similar to what we observe here with anti-M-CK antibodies. In addition, the inositol trisphosphate-activated Ca^{2+} channel was localized to the endoplasmatic reticulum located within Purkinje cell dendrites (Ross *et al.*, 1989).

Upon climbing fibre activation, a massive influx of Ca^{2+} ions occurs probably all along the proximal parts of the Purkinje cell dendrite at the abundant climbing fibre synapses. This is expected to impose high local energy requirements at those places within the dendrites where Ca^{2+} homeostasis has to be balanced. Prolongued voltage-dependent calcium conductances may also exist in pyramidal cells of the hippocampus (Ekerot and Oscarsson, 1981). Interestingly, exactly this cell type was suggested to contain anti-M-CK immunoreactivity in the human brain (Hamburg *et al.*, 1990).

Our recent experiments indicating the presence of Mi_b-CK in Purkinje neurons (unpublished) suggest that the high and fluctuating energy requirements in these cells can be met by a phosphocreatine circuit, established by the coordinate action of Mi_b-CK in the mitochondria and M-CK in the cytosol, the latter being partially bound to endoplasmic reticulum membranes (see below). The presence of the muscle-specific isoenzymes most likely represents an adaptation to the unique properties of Purkinje neurons, which are reflected in the fact that these cells express several muscle-specific proteins or muscle-specific isoforms. For example, within the chicken and mouse cerebellum, the skeletal muscle-type isoform of the ryanodine receptor seems to be almost exclusively expressed in Purkinje neurons (Kuwajiama *et al.*, 1992). Additionally, chicken Purkinje neurons represent the only non-muscle cell type in which calsequestrin, a typical protein of the sarcoplasmic reticulum, has ever been localized (Villa *et al.*, 1991; Takei *et al.*, 1992), and they contain the highest concentration of sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) in non-muscle cells (Michelangeli *et al.*, 1991). Chicken Purkinje neurons were also shown to express preferentially a specific muscle isoform of the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase, SERCA2b (Campbell *et al.*, 1993)

The presence of muscle-type M-CK in Purkinje neurons may also reflect the better suitability of M-CK, compared with other cytosolic CK isoenzymes, to associate with certain subcellular structures. Chicken M-CK, in contrast to B-CK, was shown to be associated with both the myofibrillar M-line and the sarcoplasmic reticulum, where the enzyme is functionally coupled to the myosin ATPase (Wallimann *et al.*, 1984) and the ATP-dependent Ca^{2+} pump (Rossi *et al.*, 1990), respectively.

Very recently, the Ca^{2+}-ATPase of rat skeletal muscle was shown to have preferential access to ATP generated by sarcoplasmic reticulum-bound CK (Korge *et al.*, 1993). Thus, the role M-CK plays in muscle, supplying the Ca^{2+} pump of the sarcoplasmic reticulum with ATP, may be paralleled in Purkinje neurons by (i) keeping local ATP/ADP ratios high in the vicinity of the SERCA, and by (ii) supplying this Ca^{2+}-ATPase with ATP, thereby increasing the thermodynamic efficiency of the Ca^{2+} pump (Wallimann *et al.* 1992) and thus helping to achieve rapid Ca^{2+} homeostasis in Purkinje neurons.

The fact that highly specialized brain cell types, which can be assumed to spend large amounts of energy on either K^+ homeostasis (involving BGC and astrocytes, expressing B-CK), trafficking of neurotransmitters and tricarboxylic cycle intermediates (Hertz and Peng, 1991) (involving glial cells and neurons expressing B-CK) or Ca^{2+} homeostasis (involving Purkinje neurons expressing M-CK), contain different 'cytosolic' CK isoforms is striking and deserves special attention. Future research may give further insight into the physiological relevance of CK isoenzyme diversity as well as into the specific requirements of the energy metabolism of BGC and Purkinje neurons.

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Abbreviations

Ap5A	P^1,P^5-di(adenosine-5')-pentaphosphate
B-CK	brain-type creatine kinase
BGC	Bergmann glial cell(s)
BSA	bovine serum albumin
CK	creatine kinase
EGTA	ethylene glycol tetraacetic acid
CPAGE	cellulose polyacetate gel electrophoresis
PBS	phosphate-buffered saline
GFAP	glial fibrillar acidic protein
MB-CK	heterodimer of muscle-type and brain-type creatine kinase
M-CK	muscle-type creatine kinase
MM-CK	homodimer of muscle-type creatine kinase
Mi-CK	mitochondrial creatine kinase
PMSF	phenylmethylsulphonyl fluoride
SERCA	sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase
TBS	Tris-buffered saline
TBG	1% bovine serum albumin and 0.2% gelatine in Tris-buffered saline

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