Acquisition and loss of a neuronal Ca^{2+} /calmodulin-dependent protein kinase during neuronal differentiation

(cerebellum/hippocampus/ Ca^{2+} signaling/nucleus/axon)

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ABSTRACT Calcium ions play a critical role in neural development. Insights into the ontogeny of Ca^{2+} -signaling pathways were gained by investigating the developmental expression of granule cell-enriched Ca^{2+} /calmodulin-dependent protein kinase (CaM kinase-Gr) in the cerebellum and hippocampus of the rat. Neurons of these brain regions displayed characteristic schedules by which they acquired and lost CaM kinase-Gr during differentiation. In the cerebellum, granule cells did not begin to express CaM kinase-Gr until after birth when they migrated into the granule cell layer, and this expression persisted in the adult. Purkinje cells expressed CaM kinase-Gr prenatally and lost this expression by postnatal day 14. In contrast, the granule and pyramidal cells of the hippocampus expressed the enzyme prenatally and in the adult. Moreover, CaM kinase-Gr was localized to the processes and nuclei of developing neurons. This subcellular localization together with the scheduled expression of CaM kinase-Gr can serve to regulate a developing neuron's sensitivity to Ca^{2+} at different subcellular levels.

Neuronal differentiation not only entails the acquisition of various molecular and cellular characteristics but also involves the loss of characteristics that appear only during certain stages of development (1–4). Calcium ions have been implicated in the regulation of neuronal development (5–7) and Ca^{2+} /calmodulin-dependent protein kinases have been demonstrated to be an important pathway for Ca^{2+} signaling (8, 9). In this report we describe the developmental pattern of expression of granule cell-enriched Ca^{2+} /calmodulin-dependent protein kinase (CaM kinase-Gr), which is comprised of M_r 65,000 and 67,000 polypeptides and has been localized to various neuronal populations (10). In the adult cerebellum, this enzyme was found to be concentrated in granule cells but absent from Purkinje cells (10). Because the development and circuitry of these cells have been extensively studied, we attempted to correlate the production of CaM kinase-Gr with different stages of cerebellar development. The conclusions arrived at in these studies were subsequently extended by examining the appearance of CaM kinase-Gr in the hippocampus. The present observations of developmental expression of CaM kinase-Gr typify the acquisition and loss of a Ca^{2+} -signaling pathway during development.

MATERIALS AND METHODS

Primary Antiserum. Rabbit antibodies were raised against a β -galactosidase fusion product of CaM kinase-Gr expressed in and purified from *Escherichia coli*. The antiserum was affinity-purified by adsorption to mammalian CaM kinase-Gr

isolated from rat cerebellum (10). The resulting monospecific antibody preparation was employed throughout this study.

Immunoblots. Cerebella from postnatal day (PND)-3 and adult rats were homogenized with 10 volumes of buffer containing 25 mM Hepes (pH 7.5), 2 mM EDTA, 0.1 mg of phenylmethylsulfonyl fluoride per ml, and 20 μ g of leupeptin per ml. Equivalent amounts of homogenate were electrophoresed in duplicate in SDS/10% polyacrylamide gels (11) and the proteins were electroblotted onto nitrocellulose paper (12). Blots were incubated at 4°C for 16 hr in 25 mM Tris-HCl, pH 7.5/0.15 M NaCl/1 mg of polyethylene glycol 20,000 per ml/3 mg of bovine serum albumin per ml. The blots were subsequently incubated with the affinity-purified antibody to CaM kinase-Gr in the same buffer for 1 hr at room temperature followed by extensive washing. Control blots were similarly processed but without the primary antibody. Blots were incubated together with alkaline phosphatase conjugated to goat anti-rabbit IgG for 1 hr at room temperature followed by extensive washing. Immunoreactive material was visualized by the addition of nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (13).

Immunohistochemistry. Animals at various ages from embryonic day (E)-22 to PND-14 (where PND-0 is the day of birth) were deeply anesthetized with pentobarbital (100 mg/kg) and sacrificed by cardiac perfusion with saline followed by 4% paraformaldehyde. The brains were removed, post-fixed, and immersed in 20% sucrose. Brains were frozen and sectioned sagittally at 60 μ m. Sections were incubated for 72 hr with the same affinity-purified rabbit polyclonal antiserum as used in the immunoblots. Sections were subsequently incubated with ABC reagent (Vector Laboratories) and the peroxidase label was visualized with diaminobenzidine enhanced with cobalt chloride and nickel ammonium sulfate. Control procedures (deletion of primary or secondary antibodies, neutralization with excess antigen) indicated, as observed earlier (10), that the immunoreactivity was entirely specific for CaM kinase-Gr in the adult and developing brain. Reconstruction of individual immunoreactive neurons demonstrating extensive labeling throughout its processes was performed using a neuron tracing system (Eutectic Electronics). Following light microscopic observations, selected regions of the cerebellar cortex were treated with osmium tetroxide and prepared for transmission electron microscopy. The main purpose of this study was to evaluate the pattern of expression of CaM kinase-Gr in different neuronal populations during development. Thus, rather than attempt to determine the absolute amount of enzyme by immunohistochemical methods we have focused the relative abundance

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Abbreviations: CaM kinase-Gr, granule cell-enriched Ca^{2+} /calmodulin-dependent protein kinase; CaM kinase-II, Ca^{2+} /calmodulin-dependent protein kinase-II; PND, postnatal day; E, embryonic day.

of the enzyme in different neuronal types at various development stages. However, we have observed a general correlation between immunohistochemical labeling of the enzyme and its detection by immunoblot at different stages of development.

RESULTS

Present immunohistochemical examination of adult rat cerebellum confirmed (10) the prominence of CaM kinase-Gr immunoreactivity in granule cells and its absence from Purkinje cells (Fig. 1). However, immunoreactivity could not be detected within dividing granule cell precursors in the external germinal layer or in migrating granule cells of the molecular layer (Fig. 1). The first evidence of immunoreactivity within granule cells was apparent when they reached the internal granule cell layer. Labeling within this layer increased uniformly during the second postnatal week. Consequently, it appears that the expression of CaM kinase-Gr in granule cells is a maturation-dependent monophasic process. The absence of CaM kinase-Gr from Purkinje cells of the adult cerebellum implied that these neurons employ alternative Ca^{2+} -signaling pathways. Surprisingly, CaM kinase-Gr immunoreactivity was present within immature Purkinje cells in the cerebellar anlage before birth (Fig. 1, E-22). Thus it appeared that the expression of CaM kinase-Gr was confined to Purkinje cells in the perinatal cerebellum and became restricted to granule cells in the adult cerebellum. A transition between these two stages of CaM kinase-Gr development occurred during the first two postnatal weeks in the rat. In fact, immunoreactivity was prominent in Purkinje cells until PND-7, declining thereafter to undetectable levels by PND-14. Immunoreactivity became detectable in granule cells on PND-5 and by PND-7 was expressed simultaneously by the two types of cerebellar neurons.

As expected, the immunoreactivity of the developing and adult cerebellum arose from the M_r 65,000 and 67,000 polypeptides of CaM kinase-Gr (Fig. 2). The relative abundance of these polypeptides appeared to depend on cerebellar maturation such that the M_r 65,000 component was dominant in the early postnatal cerebellum (PND-3 and PND-7), whereas both polypeptides were evident in the adult cerebellum. Additionally, it remains possible that even polypeptides with the same molecular weight may represent closely

FIG. 2. Immunoblot analysis of total cerebellar homogenates. Cerebella from PND-3 (lanes 1 and 4), PND-7 (lanes 2 and 5), and adult (lanes 3 and 6) rats were examined for their CaM kinase-Gr immunoreactivity. Lanes 1–3 were incubated with the affinity-purified antibody to CaM kinase-Gr. Control lanes (4–6) were similarly processed but without the primary antibody. The arrow denotes the M_r 65,000 polypeptide component of CaM kinase-Gr.

related though nonidentical isoenzymes that are expressed in different neuronal populations or different stages of development. The present data do not allow us to verify this possibility. As previously demonstrated (10), immunoblots confirmed the selectivity and specificity of this antibody preparation by omitting the antibody or by neutralizing it with the antigen (Fig. 2). No immunohistochemical staining was observed when the primary antibody was omitted or neutralized with the antigen.

The age-dependent differential expression of CaM kinase-Gr in particular cell populations indicated that this enzyme may represent a Ca^{2+} -signaling pathway important to neuronal differentiation. Such a possibility would be strengthened if the enzyme could mediate Ca^{2+} -signaling events at the level of the nucleus. The presence of extended stretches of glutamate residues in CaM kinase-Gr suggested

FIG. 1. Laminar distribution of CaM kinase-Gr immunoreactivity in the developing cerebellum. Different patterns of immunoreactivity were apparent in sagittal sections of cerebella from animals at various ages from E-22 to PND-14. Granule cells did not exhibit any immunoreactivity as they migrated through the molecular layer (MOL) or Purkinje cell layer (PCL) and only expressed it as they entered the granule cell layer (GCL). In contrast, Purkinje cells exhibited immunoreactivity before birth but ceased to exhibit it by PND-14.

an association with chromatin (10, 14). These conjectures were confirmed by electron microscopy, which revealed immunoreactivity in the nuclei of Purkinje cells on PND-7 as well as nuclei of mature granule cells (Fig. 3) (15).

FIG. 3. Ultrastructural localization of CaM kinase-Gr in the nuclei of developing Purkinje cells and mature granule cells. In the PND-7 Purkinje cell (A) and the mature granule cell (B), intense label is associated with the nucleus (N). Labeling (*) was also apparent in perikaryal cytoplasm of Purkinje cells (PC). Nu, nucleolus.

FIG. 4. CaM kinase-Gr immunoreactivity in sagittal sections of developing rat forebrain. CaM kinase-Gr immunoreactivity is particularly intense in the hippocampus on PND-6 and E-22 and is readily apparent in the diencephalon and cerebral cortex.

Prenatal expression of CaM kinase-Gr is not restricted to the cerebellar cortex. Particularly robust immunoreactivity was observed in the hippocampus before birth (Fig. 4). Granule and pyramidal cells of the hippocampus exhibit CaM kinase-Gr immunoreactivity as early as E-22 (Fig. 4). The expression continues postnatally (Fig. 4, PND-6) and persists in the adult (10). We have also observed similar patterns of early (Fig. 4) and persistent (10) expression of CaM kinase-Gr in regions of the cerebral cortex and diencephalon. Transient exuberant expression of CaM kinase-Gr, as exhibited by cerebellar Purkinje cells, was observed in a number of brain regions (e.g., pontine nuclei of the brainstem). Moreover, particular subsets of CaM kinase-Gr-positive neurons displayed intense immunoreactivity throughout their processes (Fig. 5).

DISCUSSION

Observations reported here lead to several conclusions that pertain to the ontogeny of Ca^{2+} -signaling pathways in developing neurons. (i) CaM kinase-Gr appears to be asynchronously produced among different neuronal subpopulations; this may reflect inherent asynchrony in the generation and maturation of distinct neuronal subtypes as well as different schedules for the induction of enzyme synthesis. (ii) Neurons regulate the synthesis and/or turnover rates of CaM kinase-Gr in a manner that yields either stable or transient expression of the kinase. Thus, the stable expression in cerebellar granule cells and hippocampal neurons contrasts with the temporary expression in Purkinje cells (Fig. 6). It remains to be determined whether the induction and repression of CaM kinase-Gr production is influenced by epigenetic variables such as neuronal activity and neurotrophic agents or whether it stems from an immutable genetic program. In any case, the acquisition and loss of a major CaM kinase by

FIG. 5. CaM kinase-Gr immunoreactive neurons from the brainstem at E-22. (A) Examples of developing neurons exhibiting intense labeling throughout their processes. (B) Reconstruction of an individual immunoreactive neuron demonstrating extensive labeling throughout its processes.

these neurons impart a developmental regulation that may alter their sensitivity to Ca^{2+} signals. (iii) Although Ca^{2+} /calmodulin-dependent protein kinase-II (CaM kinase-II) is a major component of mature hippocampal neurons (16), CaM kinase-Gr accumulation in these cells clearly precedes that of CaM kinase-II (17). Accordingly, although both enzymes can mediate Ca^{2+} signals in mature hippocampal neurons, only CaM kinase-Gr is available during earlier stages of development. The abundant prenatal expression of CaM kinase-Gr

FIG. 6. Diagrammatic representation of different developmental patterns of CaM kinase-Gr immunoreactivity. Three patterns of kinase expression were characterized in the cerebellum and hippocampus. Hippocampal neurons express CaM kinase-Gr prenatally and continue to express it into adulthood. Purkinje cells express it prenatally and cease to express it postnatally (PND-14). Cerebellar granule cells do not appear to express the enzyme until the first postnatal week and continue to express it into adulthood.

suggests that it may play a unique role in early phases of neuronal differentiation, particularly since CaM kinase-II appears to develop primarily postnatally and may be preferentially associated with later stages of neural differentiation such as synaptogenesis (17–20). (iv) The localization of CaM kinase-Gr to neuronal nuclei and developing processes parallels the axonal and dendritic localization observed in mature cerebellar granule cells (15) and indicates the potential for multiple subcellular sites of action involving nuclear Ca^{2+} signaling as well as synapsin I phosphorylation (10). These conclusions are consistent with the hypothesis that CaM kinase-Gr is a neuronal component of a Ca^{2+} -signaling pathway operating within multiple subcellular compartments at particular stages of neuronal development.

In summary, particular schedules of expression of CaM kinase-Gr in specific neuronal populations indicate that Ca^{2+} -signaling pathways can be selectively acquired or deleted during neuronal differentiation. Such a scheduled expression of Ca^{2+} -signaling pathways could modulate neuronal sensitivity to Ca^{2+} during development.

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