Evidence for Transsynaptic Regulation of Calmodulin-Dependent Cyclic Nucleotide Phosphodiesterase in Cerebellar Purkinje Cells

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Calmodulin-dependent phosphodiesterase (CaM-PDE) is selectively expressed in specific neuronal populations in adult rat brain. In cerebellar cortex, it is expressed at high levels in Purkinje cells (soma and dendrites). Climbing fiber ablation by intraperitoneal injections of 3-acetylpyridine resulted in a selective depression of cerebellar CaM-PDE expression using Western immunoblot procedures; neither calcineurin (calmodulin-dependent protein phosphatase) nor other calmodulin binding proteins, detected by biotinylated calmodulin overlays, were affected. Immunocytochemical staining of cerebellum revealed a loss of detectable CaM-PDE immunoreactivity in Purkinje cells, with no appreciable change in calcineurin immunoreactivity. Cerebral cortex was examined as a control for a direct effect of 3-acetylpyridine on CaM-PDE expression, independent of climbing fiber deafferentation. There were no detectable changes in CaM-PDE or calcineurin immunoreactivity in cortical pyramidal cells, and no changes were detected, either in Western blot analyses for CaM-PDE or calcineurin or in biotinylated calmodulin overlays. These data suggest that CaM-PDE expression in Purkinje cells is regulated transsynaptically by climbing fiber inputs.

The distribution of calmodulin-dependent phosphodiesterase (CaM-PDE) is restricted to specific neuronal populations in rat brain; in the cerebral cortex, CaM-PDE is present in pyramidal cells of layers III, V, and VI, and in the hippocampus, this enzyme is present in Cal-2 pyramidal cells (Kincaid et al., 1987). In the cerebellum, CaM-PDE immunoreactivity is prominent in the somata and dendrites of Purkinje cells and in selected deep cerebellar nuclear neurons while being absent in other types of cerebellar neurons (Fig. 1). This pattern of immunoreactivity suggests a role for Ca²⁺-regulated hydrolysis of cyclic nucleotides in Purkinje cells.

Climbing fibers provide one major excitatory input to Pur-

kinje cells (Ito, 1985), terminating on regions of the dendritic field that express CaM-PDE (Kincaid et al., 1987). Another major excitatory input, parallel fibers, terminate on spiny branchlets of the Purkinje cell dendrites; CaM-PDE does not seem to be present at high levels in this region of the dendritic field. Climbing fiber activation elicits a distinctive electrophysiologic response (complex spike), which is accompanied by a decrease in extracellular Ca²⁺ and increased extracellular K⁺ in cerebellar cortex (Stockle and Ten Bruggencate, 1980; Ito, 1985). Intradendritic recordings of complex spikes are characterized by a plateau phase (Campbell and Hesslow, 1984), which resembles the Ca²⁺-dependent plateau potential elicited by direct stimulation of dendrites (Llinás and Sugimori, 1980a, b). There is also a slowly developing hyperpolarization in dendrites after climbing fiber activation, which is not accompanied by any change in input resistance (Hounsgaard and Midtgard, 1985; Sakurai, 1987). These physiologic findings raise the possibility of a linkage between climbing fiber activation and postsynaptic influx of Ca²⁺ ions, leading to activation of Ca²⁺-dependent enzymes.

The molecular anatomy of Purkinje cells is unique in that they contain high levels of Ca²⁺-dependent enzymes such as protein kinase C, calcineurin, 28 kDa calcium binding protein (calbindin-D-28K), and an isoform of calmodulin-dependent protein kinase II (Baimbridge and Miller, 1982; Erond and Kennedy, 1985; McGuinness et al., 1985; Huang et al., 1987; Kincaid et al., 1987). This suggests that Ca²⁺ is an important second messenger in Purkinje cells. In addition, Purkinje cells selectively express cGMP protein kinase (De Camilli et al., 1984) and G-substrate (Detre et al., 1984), suggesting a regulatory role for cGMP-stimulated phosphorylation. This latter pattern of selective enzyme expression is of particular interest given the observation that cGMP is found in elevated levels in cerebellum, 10–50 times levels in other brain regions (Nathanson, 1977), suggesting a particularly important role of cGMP regulation in the cerebellar cortex.

The relationship between the distribution of climbing fiber terminals and CaM-PDE has raised the question of whether climbing fibers are involved in transsynaptic regulation of this enzyme. To test this hypothesis, climbing fibers were ablated chemically using 3-acetylpyridine (3-AP). After survival times of 2 d to 5 months, 3-AP-treated and control rats were killed and changes in the expression of calmodulin binding proteins were assessed immunocytochemically (Kincaid et al., 1987), by

Received July 5, 1988; revised Nov. 28, 1988; accepted Dec. 2, 1988.

We wish to acknowledge the excellent technical assistance of J. Kyle Krady, Carol Hoover, Robert Brucklacher, and Debra Hinton. This research was supported by PHS Grants K04 NS00891 (C.D.B.), R01 NS19850 (C.D.B.), R01 AG06377 (M.L.B.), and a grant from the International Life Sciences Institute Research Foundation (M.L.B.).

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Figure 1. Cerebellar CaM-PDE immunoreactivity was prominent in somatodendritic regions of Purkinje cells and was observed in deep cerebellar nuclei.

Western immunoblot analysis, and by analysis of biotinylated calmodulin overlays (Billingsley et al., 1985).

Materials and Methods

3-Acetylpyridine intoxication. Adult male Long-Evans and Sprague-Dawley rats were given intraperitoneal injections of 3-AP (Sigma; 75 mg/kg, 1 ml/kg total volume in saline). Three hours later, they were given an intraperitoneal injection of harmaline hydrochloride (Sigma; 15 mg/kg, 1 ml/kg total volume), which induces rhythmic activation of the inferior olive (Lamarre et al., 1971; De Montigny and Lamarre, 1973; Llinás and Volkind, 1983). One and one-half hours later, the rats received an intraperitoneal dose of niacinamide (Sigma; 300 mg/kg in a volume of 1 ml/kg), which reduces mortality (Hicks, 1955). This standard protocol produces virtually complete destruction of the inferior olive and does not affect other precerebellar structures (Llinás et al., 1975; Balaban, 1985). The rats were maintained in their home cages on *ad libitum* food and water for survival times ranging from 2 to 5 months. Most studies were performed 7 d after 3-AP treatment; previous experiments indicated that significant olivary degeneration was complete by this time.

Immunocytochemical methods. Antibody specificity and immunocytochemical methods have been described in detail previously (Kincaid

et al., 1987; Kincaid, 1988). Briefly, rats were perfused transcardially under deep pentobarbital anesthesia (75–100 mg/kg, i.p.) with PBS (0.9% NaCl in 50 mM phosphate buffer, pH 7.2–7.4) followed by 4% paraformaldehyde in 50 mM phosphate buffer (pH 7.2–7.4). After incubation overnight in perfusion fixative at 4°C, the brains were transferred to cold PBS and 50–100 μ m sections of neocortex and cerebellum were cut on an Oxford vibratome in either the transverse or horizontal plane. CaM-PDE was detected by sequential incubation in an affinity-purified primary antiserum (1:500 or 1:1000 dilution in PBS), a biotinylated goat anti-rabbit IgG antiserum and an avidin–HRP conjugate (ABC reagent, Vector Laboratories). Calcineurin was detected with an affinity-purified goat polyclonal primary antiserum, a biotinylated rabbit anti-goat IgG secondary antibody and ABC reagent. Diaminobenzidine was used as the chromogen. Sections were mounted on slides, dehydrated, cleared, and coverslipped with Permount.

Immunoblots and biotinylated calmodulin overlays. The cerebellum and neocortex were removed from control and 3-AP olivectomized rats 8 d after 3-AP treatment and homogenized in a 50 mM Tris–HCl (pH 7.4) solution containing 150 mM NaCl and 1 mM EGTA. Tissue was pooled from 3 rats to minimize interanimal variation. Crude cytosolic fractions were obtained by centrifugation. Proteins were resolved using SDS-PAGE and were transferred electrophoretically to nitrocellulose. After blocking nonspecific binding sites with 5% nonfat dry milk in Tris-

anti PDE

Figure 2. Internal control for selectivity: Effects of 3-AP olivectomy (7 d postlesion) on CaM-PDE and calcineurin expression in neocortex. *Left*, Photomicrographs of CaM-PDE immunoreactivity of layer V pyramidal cells from parietal neocortex of a representative control and 3-AP treated rat. Immunoreactivity was not affected significantly by 3-AP treatment. *Right*, Western blots and biotinylated calmodulin (*BIOCAM*) overlays (100 μg protein/lane) show that detectable neocortical levels of CaM-PDE (*PDE*), calcineurin (*CN*), or other calmodulin binding proteins were not affected by 3-AP treatment.

Figure 3. Calcineurin (CN) immunoreactivity in the cerebellum 7 d after 3-AP treatment. *Left*, Immunostained cerebellar Purkinje cells from a representative control and 3-AP-treated rat. The punctate pattern of calcineurin activity remained after 3-AP olivectomy. *Right*, Western blot and biotinylated calmodulin overlay analyses of cerebellar homogenate; calcineurin was unaffected by destruction of the inferior olive.

buffered saline containing 1 mM Ca^{2+} (TBS), the blots were reacted with biotinylated calmodulin (Biocam; 25 $\mu\text{g}/5.0$ ml TBS), a polyclonal rabbit antibody to CaM-PDE (αPDE ; 1:1000 dilution), or a polyclonal goat antibody to calcineurin (αCN ; 1:1000 dilution). Each blot analysis was performed at least twice. Biotinylated calmodulin was detected using avidin-alkaline phosphatase and a BCIP/NBT chromagen system (Billingsley et al., 1987), while immunoreactivity was detected with alkaline phosphatase conjugated to an Fab fragment of a goat anti-rabbit IgG (Promega). The BCIP/NBT chromagen system was also used to detect this conjugate.

Results

The cerebral cortex of control and 3-AP-treated rats served as an internal control for nonspecific effects of 3-AP on expression of calmodulin binding proteins. As shown in Figure 2, 3-AP treatment (7 d postinjection) did not affect either the cellular distribution of CaM-PDE in layer V pyramidal cells or levels of CaM-PDE, calcineurin, or other calmodulin binding proteins after Western blot analysis of neocortical proteins. This indicates that 3-AP treatment per se did not affect the expression

of calmodulin binding proteins in a region that does not display 3-AP-induced cell or axonal death.

In the cerebellum, 3-AP treatment did not affect the tissue distribution or levels of immunoreactive calcineurin (Fig. 3). Biotinylated calmodulin overlays revealed calmodulin binding peptides of 61, 62, 65, 75, 94, and 135 kDa in cerebellum. The 52 kDa subunit of the CaM kinase II was in much lower amounts in cerebellum compared with samples from cerebral cortex (Fig. 2); this pattern is representative of the known cerebellar isoform of this enzyme (McGuinness et al., 1985). There were no obvious differences in the pattern of calmodulin binding proteins of control and 3-AP-treated rats following injection.

When CaM-PDE immunoreactivity was examined in 3-AP-treated cerebella 7 d after injection, we observed a dramatic reduction in the tissue content of this enzyme (Fig. 4). Western immunoblot and densitometric analysis of CaM-PDE revealed a 3-fold loss of CaM-PDE in whole cerebellum after 3-AP treatment. CaM-PDE immunoreactivity of deep cerebellar nuclei was not appreciably affected by this treatment; in addition, a

Figure 4. Attenuation of Purkinje cell CaM-PDE expression 7 d after 3-AP ablation of the inferior olive. *Left*, Loss of Purkinje cell CaM-PDE immunoreactivity in a representative 3-AP-treated rat. *Middle*, Attenuation of immunoreactivity in a representative Western blot of cerebellar homogenate. Homogenates were pooled from cerebella of three 3-AP-treated or three control rats; 25 μ g was electrophoresed in each lane. *Right*, Densitometric scan (Beckman DU 8-B) of the Western blot, documented a 60–70% drop in CaM-PDE immunoreactivity.

Figure 5. Diagrammatic representation of the effects of 3-AP-induced destruction of olivary climbing fibers on the expression of CaM-PDE expression. The shaded region of the Purkinje cell reflects the presence of CaM-PDE.

few cells in the paraflocculus and flocculus remained weakly immunoreactive for CaM-PDE. These latter neuronal cell types may contribute to the residual immunoreactive CaM-PDE seen on immunoblots. This difference in CaM-PDE was not detected by the biotinylated calmodulin overlay, since CaM-PDE is a relatively minor band in total homogenate and is often obscured by the 61 kDa subunit of calcineurin and the 62 kDa subunit of CaM kinase II. Immunocytochemistry indicated that the loss of CaM-PDE following 3-AP induced olivectomy was seen as early as 2 d postinjection. No recovery was observed as long as 5 months postlesion, suggesting that climbing fiber input was necessary to maintain CaM-PDE expression. Thus, as shown schematically in Figure 5, ablation of one specific type of synaptic input is sufficient for selectively attenuating the expression of a calmodulin binding protein in Purkinje cells.

Discussion

Several studies have indicated that neurotransmitters can also affect postsynaptic gene expression at both central and peripheral sites (Kanamatsu et al., 1986; Young et al., 1986; Comb et al., 1987). For example, tyrosine hydroxylase levels are sensitive to changes in presynaptic activity and to denervation (Black et al., 1985). Similarly, nicotinic receptor stimulation of PC-12 cells induced a Ca^{2+} -dependent increase in transcription of *c-fos* and *actin* mRNA, an effect that was blocked by nicotinic antagonists (Greenberg et al., 1986). Visual experience has been reported to alter cerebral cortical expression of CaM kinase II; enzyme expression increased in macaque monkey striate cortex after monocular deprivation (Hendry and Kennedy, 1986). This report provides a similar example in the CNS. As shown schematically in Figure 5, specific chemical ablation of climbing fiber afferents to cerebellar cortex leads to a dramatic decrease in the expression of CaM-PDE only in cerebellar Purkinje cells. Other calmodulin binding proteins in cerebellum and cerebral cortex were unaffected by this chemical lesion of the inferior olive. Thus, these data imply that presynaptic activity of one class of afferents, climbing fibers, can selectively alter gene expression of calmodulin binding proteins at central postsynaptic sites. Alternatively, cerebellar CaM-PDE mRNA may be subject to specific translational control by climbing fiber input,

and the resulting loss may not be the result of directed gene regulation.

Studies of CaM kinase II have suggested that presynaptic innervation can regulate the expression of this enzyme at postsynaptic sites during development. In particular, the data suggest that expression may be either up- or down-regulated by afferent input. As mentioned previously, monocular deprivation produced an increase in CaM kinase II expression in macaque area 17 (Hendry and Kennedy, 1986). However, denervation of superior cervical ganglia produced a decrease in the CaM binding, 52 kDa major postsynaptic density protein, which is a subunit of CaM kinase II (Wu and Black, 1987). Thus, transsynaptic factors or electrical activity may modulate expression of both CaM-PDE and CaM kinase, ultimately affecting Ca^{2+} -mediated intracellular events.

Two general hypotheses can be invoked to explain these data. First, climbing fibers may have a purely trophic influence on CaM-PDE expression. For example, either synaptic contact per se or release of a trophic factor(s) may be necessary to maintain CaM-PDE expression. Alternatively, expression of CaM-PDE may be functionally coupled to neurotransmission. This selective effect is particularly impressive in light of the relatively small contribution of climbing fiber terminals to the total synaptic surface area of Purkinje cells (Larramendi and Victor, 1967; Palay and Chan-Palay, 1985). In this regard, it is compelling that CaM-PDE, like climbing fibers, is distributed only in somata and nonspiny dendritic branches of Purkinje cells. This suggests a close relationship between climbing fiber function and regional expression of a subset of enzymes within the postsynaptic cells. The apparent selectivity of the effect of climbing fiber deafferentation on the expression of one CaM binding protein is intriguing, given the relatively high expression of CaM and other CaM binding proteins in these cells. However, such an effect would imply that climbing fibers are intimately involved in both short- and long-term regulation of interactions between postsynaptic calcium fluxes and cyclic nucleotide levels in Purkinje cells.

The influence of climbing fiber innervation on CaM-PDE expression in Purkinje cells raises the testable hypothesis that the enzyme is involved in internal signal integration subserving

the reported long-term interactions between climbing fiber and parallel fiber synaptic inputs. Ito and colleagues (1982) reported that conjunctive stimulation of climbing fibers and vestibular mossy fibers produced a persistent depression of Purkinje cell responsiveness to vestibular mossy fiber inputs. Since transmission was not affected at other sites, they argued that depressed parallel fiber–Purkinje cell transmission was responsible for this effect. This inference has been confirmed by Ekerot and Kano (1985) after conjunctive parallel fiber and climbing fiber stimulation and by Sakurai (1987) in cerebellar slice preparations. The latter findings indicate that parallel fiber activation alone potentiates subsequent fiber EPSPs in postsynaptic Purkinje cells, while conjunctive climbing fiber and parallel fiber stimulation produces a depression of parallel fiber transmission. By contrast, climbing fiber activation alone has no effect. Since Purkinje cell responses to climbing fiber activation include a plateau potential that is associated with a Ca^{2+} influx (Llinás and Sugimori, 1980a, b; Campbell and Hesslow, 1984), the close association of CaM-PDE expression and climbing fiber innervation suggests investigations of interactions between regional cyclic nucleotide metabolism and parallel fiber activation in these heterosynaptic interactions.

An influence of climbing fiber innervation on CaM-PDE expression probably reflects only one component of complex postsynaptic mechanisms that are either regulated by intracellular Ca^{2+} or regulate cyclic nucleotide levels. Since other Ca^{2+} -binding (Baimbridge and Miller, 1982) and CaM dependent enzymes are present in Purkinje cells (Erondy and Kennedy, 1985; Kincaid et al., 1987), the ion fluxes associated with climbing fiber activation have the potential to influence multiple enzyme cascades. Sakurai's (1987) demonstration that the temporal relationship between climbing fiber and parallel fiber inputs either has no effect on or can potentiate or depress subsequent parallel fiber EPSPs of individual Purkinje cells suggests that there is an intricate intracellular integrative system mediating long-term, heterosynaptic interactions. Although pharmacologic climbing fiber activation and direct application of excitatory amino acids (or their antagonists) have been shown to affect cerebellar cGMP levels (e.g., Biggo and Guidotti, 1976; Wood et al., 1982, 1987), these events have been observed under highly restricted, artificial experimental conditions. Thus, it is premature to speculate on the implications of these findings *in vivo*.

The selective transsynaptic regulation of CaM-PDE expression in cerebellum raises the more general issue of the role of synaptic inputs in CaM-PDE regulation in other neuronal populations. Since CaM-PDE is selectively expressed in neocortex, hippocampus, olfactory cortex, and thalamus (Kincaid et al., 1987), it is important to determine whether analogous transsynaptic controls exist for each site. This question is of particular import during early development, when synaptogenesis may activate or attenuate expression of CaM-PDE to establish and maintain an adult pattern of expression.

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