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Localization of cyclic GMP-dependent protein kinase and substrate in mammalian cerebellum

(protein phosphorylation/photoaffinity labeling/Purkinje cell/neurological mutant mice)

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ABSTRACT **The regional and cellular distribution of guanosine 3′,5′-cyclic monophosphate (cGMP)-dependent protein kinase (ATP:protein phosphotransferase, EC 2.7.1.37) in mammalian brain was examined by use of the photoaffinity label 8-azidoinosine 3′,5′-cyclic monophosphate. Of the regions examined, cerebellum had by far the highest concentration of this enzyme. The cellular localization of cGMP-dependent protein kinase within the cerebellum was determined by examination of mutant mice missing specific types of cerebellar neurons. Mutant mice lacking Purkinje cells had greatly reduced amounts of cGMP-dependent protein kinase, whereas the loss of another cell type, granule cells, did not reduce cGMP-dependent protein kinase levels. By using the same strains of mutant mice, a 23,000-dalton soluble cerebellar substrate for cGMP-dependent protein kinase was also shown to be enriched in Purkinje cells. In contrast, the concentration of type I 3′,5′-cyclic AMP-dependent protein kinase in the cerebellum was unaffected by the absence of Purkinje cells and only slightly reduced by the absence of granule cells. The enrichment in Purkinje cells of the cGMP-dependent protein kinase and its substrate suggests an important role for cGMP and cGMP-dependent protein phosphorylation in the function of this type of neuronal cell.**

Considerable evidence now suggests that guanosine 3′,5′-cyclic monophosphate (cGMP) may play a role in neuronal function (1–3) and that many effects of this nucleotide are mediated by activation of cGMP-dependent protein kinase (ATP:protein phosphotransferase, EC 2.7.1.37) (4). However, the distribution of this enzyme in neuronal tissue has not been closely examined. Cerebellum is the only neuronal tissue that has been reported to contain a high concentration of cGMP-dependent protein kinase (5–7) and of a substrate for this enzyme (8). In addition, the activity of cGMP-dependent protein kinase has been reported to be reduced in the cerebellum of several strains of mutant mice deficient in Purkinje cells (9). In the present study, we have determined the concentration of this enzyme in various regions of brain. We have also studied the cellular localization of cGMP-dependent protein kinase and a substrate (8) for this enzyme within the cerebellum, using several strains of mutant mice deficient in specific types of cerebellar neurons.

MATERIALS AND METHODS

Materials. The ^{32}P-labeled photoaffinity label 8-azidoinosine 3′,5′-cyclic monophosphate (8-N_3-[^{32}P]cIMP) was synthesized from 8-N_3-cyclic [^{32}P]AMP (New England Nuclear) by the method of Casnellie *et al.* (10). Material of greater than 95% radiochemical purity was obtained, and purity was checked immediately prior to use by thin-layer chromatography (10).

Mutant Mice. Neurological mutant mice C57BL/6J Purkinje cell degeneration (PCD; *pcd/pcd*), C3HeB/FeJ nervous (*nr/nr*), B6CBA-A$^{w\text{-}J}$/A weaver (*wv/wv*), C57BL/6J staggerer (*sg/sg*), and their respective age-matched controls were obtained from Jackson Laboratories. Both PCD and nervous mutants selectively lose greater than 90% of cerebellar Purkinje cells by the age of 2 months, with no significant reduction in the numbers of other cell types (11, 12). In weaver mutants, granule cells are essentially absent by the age of 3 weeks (13, 14), with no significant loss of other types (15). The staggerer mutation results in the loss of virtually all cerebellar granule cells by the age of 3 weeks (16) and a large deficit of Purkinje cells (17). Nervous, PCD, and staggerer are autosomal recessive mutations; heterozygous animals are phenotypically normal (11, 12, 16). Heterozygous weavers lose a small fraction of cerebellar granule cells, but are behaviorally normal (14). Homozygous recessive animals possessing cerebellar deficits were identified by their locomotor abnormalities. Control heterozygous and homozygous wild-type animals were behaviorally indistinguishable and are collectively referred to as +/? animals of the appropriate strain. PCD animals were killed at 3 months of age, nervous and weaver at 2–2.5 months, and staggerer at 21–28 days. Frozen cerebella of X-irradiated rats were a generous gift of Hermes Yeh.

Preparation of Tissue Extracts for Photoaffinity Labeling. Adult cats, weighing 4–4.5 kg, were anesthesized with pentobarbital at 30 mg/kg intravenously and ventilated through a tracheotomy tube after administration of Flaxedil at 3 mg/kg. Various locations in the brain were biopsied and each specimen was immediately immersed in 10 ml of ice-cold "homogenization buffer" containing 10 mM Hepes (pH 7.0), 5 mM 2-mercaptoethanol, 1 mM EDTA, 30 μM phenylmethylsulfonyl fluoride, 2% (vol/vol) ethanol, and 0.25 M sucrose. Mice were killed by cervical dislocation and individual cerebella were placed on ice in homogenization buffer. Brain microvessels were prepared from whole rabbit brain by the technique of Nathanson and Glaser (18) and suspended in homogenization buffer. All subsequent steps were carried out at 4°C. The tissue was homogenized by hand in 4–6 vol of homogenization buffer, and the homogenate was centrifuged for 45 min at 150,000 × *g*. The resulting cytosol was used immediately for photoaffinity labeling or pretreated by a 10-min incubation at 30°C with 50 μg of beef heart phosphodiesterase (Boehringer Mannheim)

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Abbreviations: cGMP, guanosine 3′,5′-cyclic monophosphate; cAMP, adenosine 3′,5′-cyclic monophosphate; 8-N_3-cIMP, 8-azidoinosine 3′,5′-cyclic monophosphate; PCD, Purkinje cell degeneration mouse strain; R-I and R-II, regulatory subunits of the type I and type II cyclic AMP-dependent protein kinases, respectively; Dal, dalton; 23-kDal G-substrate, 23,000-dalton substrate for cyclic GMP-dependent protein kinase.

to destroy any remaining endogenous cyclic nucleotide. Protein in the cytosol samples was assayed by the method of Bradford (19) with bovine gamma globulin as the standard.

Photoaffinity Labeling. Photoaffinity labeling with 8-N_3-[^{32}P]cIMP was performed by minor modification of the procedure of Casnellie *et al.* (20). The reaction mixture contained, in a final volume of 100 μl, 50 mM Hepes (pH 7.5), 1 μM 8-N_3-[^{32}P]cIMP (10–25 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels), 0.2 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid, 5 mM 2-mercaptoethanol, 1 mM 3-isobutyl-1-methylxanthine, cGMP where indicated, and 100–400 μg of cytosol protein. Preincubations were carried out for 60 min at 0°C and the samples were then irradiated for 10 min at 0°C with a Mineralite UVS lamp at a distance of 3 cm. After photolysis, the samples were subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis, and the gels were dried and autoradiography was performed as described (20). Proteins of known molecular weight (phosphorylase A, 92,500; bovine serum albumin, 67,000; ovalbumin, 43,500; α-chymotrypsinogen, 23,650) were used as standards for $NaDodSO_4$/polyacrylamide gel electrophoresis. The absolute amount of 8-N_3-[^{32}P]cIMP covalently incorporated was determined by cutting the appropriate bands from the dried gel and counting by liquid scintillation spectrometry. The amount of 8-N_3-[^{32}P]cIMP incorporated was linear up to 600 μg of cytosol protein in the incubation mixture.

Efficiency of Incorporation of 8-N_3-[^{32}P]cIMP. cGMP-dependent protein kinase was purified from bovine lung according to Walter *et al.* (21). The cGMP-dependent protein kinase appeared to be homogenous as judged by densitometric scanning of Coomassie brilliant blue R-stained gels of the enzyme preparation. Specific activity of the enzyme, assayed according to Flockerzi *et al.* (22), with histone H2b (Worthington) as a substrate, was 3.2 μmol of ^{32}P transferred per min per mg of protein. Under standard photoaffinity labeling conditions, the incorporation of 8-N_3-[^{32}P]cIMP into the purified enzyme was 0.182 ± 0.041 mol per mol of holoenzyme (145,000-dalton dimer) (mean ± SD, $n = 7$). Because addition of cytosol protein to the incubation mixture did not alter the observed efficiency of incorporation into the purified enzyme, absolute values for cGMP-dependent protein kinase holoenzyme in various regions of brain have been calculated by assuming the same efficiency of incorporation of 8-N_3-[^{32}P]cIMP into the cytosol enzyme.

Type I and type II cyclic AMP (cAMP)-dependent protein kinase regulatory subunits (R-I and R-II, respectively) (23, 24) are also covalently labeled by the photoaffinity label 8-N_3-[^{32}P]cIMP (20). The efficiency of incorporation of 8-N_3-[^{32}P]cIMP into type I and type II cAMP-dependent protein kinases was determined to be 0.218 and less than 0.04 mol per mol of holoenzyme (tetramer), respectively. This difference in the efficiency of incorporation of the photoaffinity label into the two types of regulatory subunit agrees with the higher affinity for cIMP reported for R-I (24). In most tissues, the concentration of regulatory subunit is an accurate measure of the concentration of the cAMP-dependent protein kinase holoenzyme (25–27). Therefore, we have used the photoactivated incorporation of 8-N_3-[^{32}P]cIMP into R-I as a measure of the concentration of type I cAMP-dependent protein kinase. (The efficiency of incorporation of 8-N_3-[^{32}P]cIMP into R-II was too low to allow accurate measurement of the type II cAMP-dependent protein kinase.)

Assay of 23,000-Dalton (23-kDal) Substrate of cGMP-Dependent Protein Kinase (23-kDal G-Substrate). Cerebella of mutant and control mice were rapidly dissected and placed in ice-cold 0.32 M sucrose. All subsequent operations were carried out at 4°C. Cerebella were transferred to, and homogenized in, 5 or 10 vol of a solution containing 10 mM Hepes (pH 7.4), 0.1 mM EDTA, 30 mM 2-mercaptoethanol, 100 μM phenylmethylsulfonyl fluoride. The homogenates were centrifuged at 27,000 × *g* for 20 min. An aliquot (0.3 ml) of the supernatant was desalted on a 2.5-ml bed of Sephadex G-25, previously equilibrated with buffer of the same composition, using a modified centrifuge desalting technique (28). Protein in the eluate was assayed by the method of Lowry *et al.* (29). The desalted supernatant was subjected to phosphorylation in a final volume of 100 μl containing 175 μg of cytosol protein, 10 mM Hepes (pH 7.4), 0.3 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid, 1 μM cGMP, 1 mM 3-isobutyl-1-methylxanthine, 10 mM $MgCl_2$, 4 μg of purified cGMP-dependent protein kinase, and 25 μM [γ-^{32}P]ATP (2–3 Ci/mmol). The phosphorylation reaction was carried out at 30°C for 1.5 min. The reaction was stopped by addition of a $NaDodSO_4$-containing stop solution, and $NaDodSO_4$/polyacrylamide gel electrophoresis and autoradiography were carried out as described (8). Relative incorporation of ^{32}P into 23-kDal G-substrate, determined by scanning of the autoradiograms with a Joyce–Loebl Model MKIIIC microdensitometer, was used as a measure of the amount of this substrate.

RESULTS

Photoaffinity labeling with 8-N_3-[^{32}P]cIMP of cytosol proteins from cat cerebellum resulted in the covalent incorporation of radioactivity into major protein bands of molecular weights 74,000, 55,000–53,000, and 47,000 (Fig. 1). The heavily labeled band at 74,000 corresponds to the monomer of cGMP-dependent protein kinase (20); the photoactivated incorporation of 8-N_3-[^{32}P]cIMP into this band was essentially abolished by the addition of 5 μM cGMP. The proteins with molecular weights of 55,000–53,000 and 47,000 are R-II and R-I, respectively, which are also covalently labeled by 8-N_3-cIMP (20). [R-II is

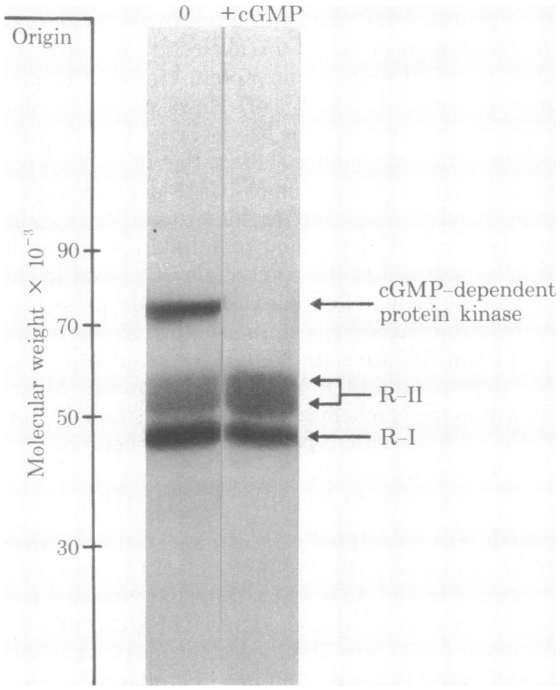

FIG. 1. Autoradiograph showing photoactivated incorporation of 8-N_3-[^{32}P]cIMP into cytosol proteins of cat cerebellum. Photoaffinity labeling was carried out under standard conditions in the absence or presence of 5 μM cGMP, and the proteins (300 μg per lane) were separated by $NaDodSO_4$/polyacrylamide gel electrophoresis.

Table 1. Concentration of cGMP-dependent protein kinase holoenzyme in cytosol of various brain regions of the cat

Brain region	cGMP-dependent protein kinase, pmol/mg protein
Cerebellar nuclei	1.73
Cerebellar vermis (cortex)	1.20
Cerebellar hemisphere (cortex)	0.90
Choroid plexus	0.34
Medulla	0.11
Temporal cortex	0.08
Hippocampus	0.05
Tectum	0.04

Photoaffinity labeling was carried out, using 200–400 μg of cytosol protein, under standard conditions, and the absolute amount of 8-N_3-[^{32}P]cIMP incorporated was corrected for the efficiency of incorporation as described.

divided into two bands upon NaDodSO$_4$/polyacrylamide gel electrophoresis because the phosphorylated form of the subunit migrates slightly more slowly than the nonphosphorylated form (30–32).]

The photoactivated incorporation of 8-N_3-[^{32}P]cIMP was used to assay cGMP-dependent protein kinase in various regions of the cat brain (Table 1). Cerebellum contained much more cGMP-dependent protein kinase than any other brain region examined. Similar results were obtained with rat and rabbit brain (data not shown). Regions of cat brain that contained low but significant amounts of cGMP-dependent protein kinase (i.e., between 0.02 and 0.04 pmol/mg of protein) were: frontal cortex, occipital cortex, corpus callosum, basal ganglia, thalamus, hypothalamus, olfactory bulb, pons, and pituitary. The concentration of cGMP-dependent protein kinase in microvessels isolated from whole rabbit brain was 0.16 pmol/mg of protein. Addition of cytosol from brain regions low in cGMP-dependent protein kinase did not reduce incorporation of 8-N_3-cIMP into the 74,000-Dal cerebellar cytosol protein, indicating that the relatively low incorporation of 8-N_3-cIMP found in brain regions other than cerebellum was not due to the presence of endogenous inhibitors that prevented 8-N_3-cIMP binding. No increase in 8-N_3-cIMP incorporation into the 74,000-Dal monomer of cGMP-dependent protein kinase was observed, in cytosol from various brain regions, as a result of preincubation with phosphodiesterase under conditions in which complete destruction of added cGMP was observed, indicating that little or no endogenous cGMP remained bound to the enzyme under the conditions used. The distribution of cGMP-dependent protein kinase observed in various brain regions (Table 1) is in excellent agreement with data on the distribution of cGMP-dependent protein kinase obtained by radioimmunoassay (U. Walter, P. Miller, and P. Greengard, unpublished observations).

By utilizing the technique of photoaffinity labeling, and strains of mutant mice deficient in specific types of cerebellar neurons, it was possible to determine the localization of cGMP-dependent protein kinase within the cerebellum. Photoaffinity labeling of cytosol from cerebella of mice lacking Purkinje cells (PCD, nervous) revealed large reductions in the level of cGMP-dependent protein kinase (Fig. 2), suggesting that Purkinje cells are highly enriched in this enzyme. In homozygous recessive PCD mice (*pcd/pcd*), which have an almost total absence of Purkinje cells, the cerebellar cGMP-dependent protein kinase was reduced to less than 4% of that seen in the normal animal when the data were expressed either on the basis of enzyme per mg of protein or on the basis of enzyme per total cerebellum (Table 2). In contrast, the concentration of type I cAMP-dependent protein kinase was not reduced in the *pcd/pcd* animal. (The loss of type I cAMP-dependent protein kinase per total cerebellum in the *pcd/pcd* animals was due to the reduction in cerebellar mass seen in the mutant animals.)

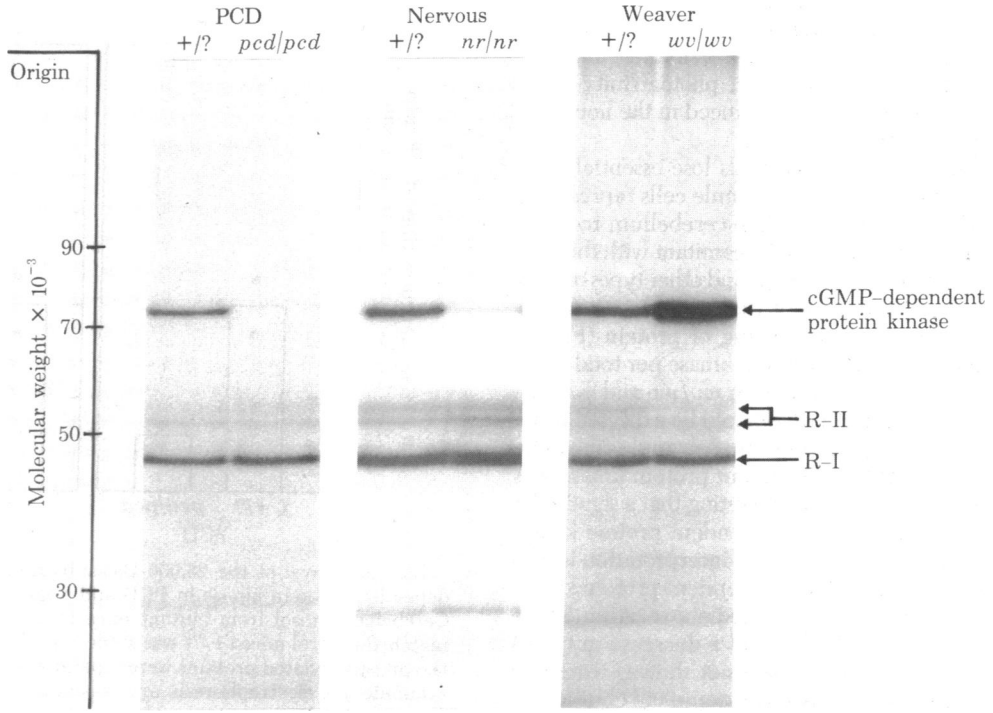

FIG. 2. Autoradiograph showing photoactivated incorporation of 8-N_3-[^{32}P]cIMP into cytosol proteins of individual cerebella from various types of neurological mutant mice. Photoaffinity labeling was carried out under standard conditions and the proteins (200 μg per lane) were separated by NaDodSO$_4$/polyacrylamide gel electrophoresis. Results obtained with homozygous mutants (*pcd/pcd, nr/nr, wv/wv*) and age-matched controls (+/?) are shown.

Table 2. Levels of cGMP-dependent protein kinase holoenzyme and of type I cAMP-dependent protein kinase holoenzyme in cerebellar cytosol of various neurological mutant mice

Mutation	Genotype	Protein kinase, pmol/mg protein		Cerebellar wt, % of control	Protein kinase, pmol/cerebellum	
		cGMP-dependent	Type I cAMP-dependent		cGMP-dependent	Type I cAMP-dependent
PCD	+/?	0.60 ± 0.08	0.48 ± 0.03	100	0.64 ± 0.11	0.51 ± 0.13
	pcd/pcd	0.02 ± 0.01	0.49 ± 0.07	52	0.01 ± 0.01	0.26 ± 0.07
Nervous	+/?	0.55 ± 0.01	0.70 ± 0.14	100	0.45 ± 0.05	0.56 ± 0.05
	nr/nr	0.08 ± 0.01	0.97 ± 0.06	67	0.04 ± 0.01	0.44 ± 0.03
Weaver	+/?	0.48 ± 0.13	0.42 ± 0.03	100	0.34 ± 0.10	0.38 ± 0.04
	wv/wv	1.30 ± 0.41	0.30 ± 0.10	37	0.43 ± 0.08	0.08 ± 0.02

Photoaffinity labeling was carried out, using 150–300 μg of cytosol protein, under standard conditions and the absolute amount of 8-N_3-[^{32}P]cIMP incorporated was corrected for the efficiency of incorporation as described. Data represent the mean ± SD for four to six samples of each genotype.

The homozygous recessive nervous mutants (*nr/nr*), which lose 90% of their cerebellar Purkinje cells, showed an 85% reduction in the concentration of cGMP-dependent protein kinase as compared to phenotypically normal animals (Table 2). The concentration of type I cAMP-dependent protein kinase was slightly increased in these mutant animals; the reason for this increase is unknown. A greater than 70% reduction in the amount of cGMP-dependent protein kinase in the cerebellum, with only a slight decrease in the amount of type I cAMP-dependent protein kinase, was found in the staggerer mutant (data not shown), which loses virtually all of its granule cells (16) but also has a significant (60–90%) reduction in the number of Purkinje cells (17).

Addition of cerebellar cytosol from homozygous recessive mutants did not reduce incorporation of 8-N_3-cIMP into the 74,000-Dal protein present in cerebellar cytosol from control mice, indicating that the relatively low incorporation of 8-N_3-cIMP found in the mutants was not due to the presence of endogenous inhibitors that prevented 8-N_3-cIMP binding. Moreover, experiments with phosphodiesterase, analogous to those described above, indicated that the low incorporation of 8-N_3-cIMP observed in the Purkinje cell homozygous mutants was not due to an increase in cerebellar cGMP in these animals, which could have prevented binding of the photoaffinity label; in fact, cGMP levels are significantly reduced in the homozygous recessive nervous animals (33).

Homozygous recessive weaver animals lose essentially all cerebellar granule cells, and because granule cells represent a large portion of the cerebellar mass, the cerebellum in these animals is greatly reduced in weight. Concomitant with this loss, there is a relative enrichment of Purkinje and other types of cells in the remaining tissue and a more than 2-fold increase in cGMP-dependent protein kinase per mg of protein (Fig. 2, Table 2). The cGMP-dependent protein kinase per total cerebellum was not significantly different for *wv/wv* and +/? animals (Table 2); indeed, no difference would be expected if the enzyme is located in cell type(s) other than granule cells. The concentration of type I cAMP-dependent protein kinase was slightly reduced in *wv/wv* animals, suggesting that a significant portion of cerebellar type I cAMP-dependent protein kinase may be present in the granule cells. This interpretation is supported by the data on type I cAMP-dependent protein kinase obtained with the staggerer mutant cited above. An increase in cGMP-dependent protein kinase and a decrease in type I cAMP-dependent protein kinase (data not shown) were also observed in cerebella from rats in which complete elimination of cerebellar granule cells was achieved by postnatal X-irradiation (34).

Previous work from this laboratory (8) demonstrated that a specific substrate for cGMP-dependent protein kinase (23-kDal G-substrate) exists in the cytosol of rabbit cerebellum. Mouse cerebellum contains a similar 23-kDal G-substrate, although the level in mouse is only 10–20% of that in rabbit (unpublished observations). Because the cGMP-dependent protein kinase in mice appeared to be highly concentrated in Purkinje cells, it was of interest to determine if the 23-kDal G-substrate showed a similar distribution. The apparent amount of the 23-kDal G-substrate in PCD mutants was found to be 70% lower than that in age-matched control mice (Fig. 3), suggesting that Purkinje cells are highly enriched in this substrate. In the homozygous recessive weaver mutants, the level of the 23-kDal G-substrate was 2-fold higher than that in the control mice. Purified 23-kDal G-substrate (from rabbit cerebellum) was found to be phosphorylated by purified cGMP-dependent protein kinase to an equal extent in the presence of cytosol from mutant and control mice (unpublished observations), suggesting that the observed differences in incorporation of ^{32}P into the endogenous 23-kDal G-substrate were not due to differences in amounts of inhibitors of phosphorylation, of phosphatases, or of proteolytic enzymes degrading the substrate. The 2-fold increase in the 23-kDal G-substrate in the weaver mutant was

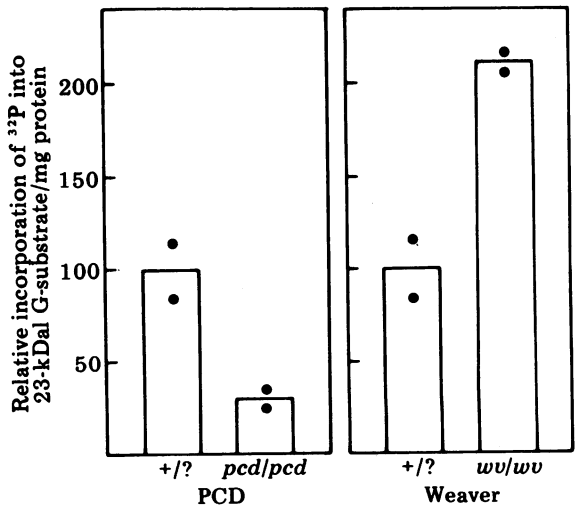

FIG. 3. Level of the 23,000-Dal substrate protein for cGMP-dependent protein kinase in PCD and weaver cerebellar mutants. Cerebellar cytosol from mutant mice (*pcd/pcd, wv/wv*) and age-matched control mice (+/?) was subjected to phosphorylation, and the phosphorylated proteins were separated by NaDodSO$_4$/polyacrylamide gel electrophoresis and visualized by autoradiography. Substrate levels were measured by scanning the autoradiograms with a densitometer. Each bar represents the average of two samples, with two cerebella pooled per sample. ●s, Actual values for the pooled cerebella. The mean value of the controls for each strain of mice was arbitrarily set at 100 units.

similar to the increase in cGMP-dependent protein kinase observed in this mutant and was presumably due to the relative enrichment of other cell types resulting from the loss of granule cells.

DISCUSSION

The reduction in the concentration of cGMP-dependent protein kinase found in the PCD and nervous mutants suggests that over 95% of cerebellar cGMP-dependent protein kinase is localized in Purkinje cells. The decrease in the amount of this enzyme found in staggerer mutants and the enrichment of the enzyme found in weaver mutants and in X-irradiated rats is compatible with this idea. Moreover, recent immunocytochemical studies using antibodies to cGMP-dependent protein kinase support the conclusion that the enzyme is highly concentrated in Purkinje cells of the cerebellum (P. De Camilli, F. E. Bloom, S. M. Lohmann, U. Walter, and P. Greengard, unpublished observations). A 70% reduction in the histone kinase activity of cGMP-dependent protein kinase was previously reported for the nervous mutant (9). The reduction in cGMP-dependent protein kinase observed in the present studies was not accompanied by a similar decrease in the concentration of type I cAMP-dependent protein kinase. In fact, an increased concentration of cerebellar type I cAMP-dependent protein kinase was found in *nr/nr* mice that had lost almost all of their cerebellar cGMP-dependent protein kinase. This increase could be due to an increase in some other neuronal or nonneuronal element in the cerebellum subsequent to the degeneration of the Purkinje cells. It has been reported that cAMP levels are normal in the cerebellum of this mutant (33).

Assay of cGMP-dependent protein kinase by photoaffinity labeling indicated that the concentration of this enzyme was much higher in cerebellum than in other brain regions. However, some cGMP-dependent protein kinase was found in all brain regions examined. Interestingly, the concentration of this enzyme remaining in the cerebella of mutant mice lacking Purkinje cells, 0.02 pmol/mg of protein, was similar to the level of this enzyme in most other regions of the brain. An 8-fold higher concentration of cGMP-dependent protein kinase (0.16 pmol/mg of protein) was found in microvessels isolated from brain. It is probable, therefore, that a portion of the cGMP-dependent protein kinase observed in brain cytosol is derived from blood vessels. However, because microvessels make up less than 1% of the brain mass (35), it would seem that a maximum of 10% of the cGMP-dependent protein kinase found in brain cytosol could have been derived from this source.

Changes in the level of the 23-kDal G-substrate qualitatively paralleled changes in the concentration of cGMP-dependent protein kinase in both PCD and weaver mutant mice. The reduction in the apparent amount of 23-kDal G-substrate found in the PCD mutant mice was substantial (70%) but less than the reduction observed in the concentration of the enzyme. However, preliminary experiments indicate that the 30% of substrate apparently remaining in the PCD mutants may be largely attributable to a contaminating phosphoprotein in the 23-kDal region of the gels used to measure substrate levels. Thus, the true reduction in the amount of the 23-kDal G-substrate in the PCD mutant mice may have been much greater than 70%.

In conclusion, it seems very likely that both cerebellar cGMP-dependent protein kinase and the 23-kDal G-substrate are highly enriched in Purkinje cells. The uniquely high concentration of these two elements of the cGMP-dependent protein phosphorylation system in Purkinje cells suggests that cGMP-dependent phosphorylation of specific protein(s) may have an important function in this type of neuronal cell.

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1. Bloom, F. E. (1975) *Rev. Physiol. Biochem. Pharmacol.* **74,** 1–103.
2. Greengard, P. (1976) *Nature (London)* **260,** 101–108.
3. Nathanson, J. A. (1977) *Physiol. Rev.* **57,** 157–256.
4. Greengard, P. (1978) *Science* **199,** 146–152.
5. Greengard, P. & Kuo, J. F. (1970) in *Advances in Biochemical Psychopharmacology,* eds. Greengard, P. & Costa, E. (Raven, New York), Vol. 3, pp. 287–306.
6. Hofmann, F. & Sold, G. (1972) *Biochem. Biophys. Res. Commun.* **49,** 1100–1107.
7. Takai, Y., Nishiyama, K., Yamamura, H. & Nishizuka, Y. (1975) *J. Biol. Chem.* **250,** 4690–4695.
8. Schlichter, D. J., Casnellie, J. E. & Greengard, P. (1978) *Nature (London)* **273,** 61–62.
9. Bandle, E. & Guidotti, A. (1978) *Brain Res.* **156,** 412–416.
10. Casnellie, J. E., Ives, H. E., Jamieson, J. D. & Greengard, P. (1980) *J. Biol. Chem.* **255,** 3770–3776.
11. Landis, S. C. (1973) *J. Cell Biol.* **57,** 782–797.
12. Landis, S. C. & Mullen, R. J. (1978) *J. Comp. Neurol.* **177,** 125–144.
13. Rakic, P. & Sidman, R. L. (1973) *J. Comp. Neurol.* **152,** 103–132.
14. Roffler-Tarlov, S. & Sidman, R. L. (1978) *Brain Res.* **142,** 269–283.
15. Rakic, P. & Sidman, R. L. (1973) *J. Comp. Neurol.* **152,** 133–162.
16. Landis, D. M. D. & Sidman, R. L. (1978) *J. Comp. Neurol.* **179,** 831–864.
17. Herrup, K. & Mullen, R. J. (1979) *Brain Res.* **172,** 1–12.
18. Nathanson, J. A. & Glaser, G. H. (1979) *Nature (London)* **278,** 567–569.
19. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254.
20. Casnellie, J. E., Schlichter, D. J., Walter, U. & Greengard, P. (1978) *J. Biol. Chem.* **253,** 4771–4776.
21. Walter, U., Miller, P., Wilson, F., Menkes, D. & Greengard, P. (1980) *J. Biol. Chem.* **255,** 3757–3762.
22. Flockerzi, V., Spiechermann, N. & Hofmann, F. (1978) *J. Biol. Chem.* **253,** 3395–3399.
23. Reimann, E. M., Walsh, D. A. & Krebs, E. G. (1973) *J. Biol. Chem.* **246,** 1986–1995.
24. Walter, U., Lohmann, S. M., Sieghart, W. & Greengard, P. (1979) *J. Biol. Chem.* **254,** 12235–12239.
25. Corbin, J. D., Sugden, P. H., Lincoln, T. M. & Keeley, S. L. (1977) *J. Biol. Chem.* **252,** 3854–3861.
26. Hofmann, F., Bechtel, P. J. & Krebs, E. G. (1977) *J. Biol. Chem.* **252,** 1441–1447.
27. Lohmann, S. M., Walter, U. & Greengard, P. (1978) *J. Cyclic Nucleotide Res.* **4,** 445–452.
28. Helmerhorst, E. & Stokes, G. B. (1980) *Anal. Biochem.* **104,** 130–135.
29. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
30. Hofmann, F., Beavo, J. A., Bechtel, P. J. & Krebs, E. G. (1975) *J. Biol. Chem.* **250,** 7795–7801.
31. Walter, U. & Greengard, P. (1978) *J. Cyclic Nucleotide Res.* **4,** 437–444.
32. Rangel-Aldao, R., Kupiec, J. W. & Rosen, O. M. (1979) *J. Biol. Chem.* **254,** 2499–2508.
33. Mao, C. C., Guidotti, A. & Landis, S. (1975) *Brain Res.* **90,** 335–339.
34. Altman, J. & Anderson, W. J. (1972) *J. Comp. Neurol.* **146,** 355–406.
35. Peroutka, S. J., Moskowitz, M. A., Reinhard, J. F., Jr. & Snyder, S. H. (1980) *Science* **208,** 610–612.