LOCALIZATION IN MAMMALIAN BRAIN OF G-SUBSTRATE, A SPECIFIC SUBSTRATE FOR GUANOSINE 3',5'-CYCLIC MONOPHOSPHATE-DEPENDENT PROTEIN KINASE¹

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Abstract

The regional and cellular distribution of G-substrate, a 23,000-dalton protein substrate specific for guanosine 3',5'-cyclic monophosphate-dependent protein kinase, has been examined in mammalian brain using immunoprecipitation, radioimmunoassay, and peptide-mapping techniques. In rabbit brain, G-substrate was found to be highly concentrated in the cerebellum. The concentration of G-substrate in cerebellar cytosol was 27.2 pmol/mg. The concentrations of G-substrate in cortex, hippocampus, and caudate were only 1 to 2% of that found in cerebellum. Studies of neurological mutant mice lacking either Purkinje cells (PCD and nervous) or granule cells (weaver) suggested that, within the cerebellum, G-substrate is localized almost exclusively in Purkinje cells. A phosphoprotein present in noncerebellar brain regions, which co-migrated with G-substrate on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, was shown by peptide mapping to consist predominantly of phosphatase inhibitor-1. Phosphatase inhibitor-1, a potent inhibitor of protein phosphatase-1, is known to share several physicochemical properties with G-substrate. In contrast to the results obtained with G-substrate, the concentration of phosphatase inhibitor-1 was significantly lower in cerebellum than in other major brain regions. These and other data suggest that G-substrate may be a Purkinje cell-specific protein phosphatase inhibitor.

Considerable evidence suggests that guanosine 3',5'-cyclic monophosphate (cyclic GMP) plays a role in neuronal function and that many of the effects of this nucleotide are mediated by activation of cyclic GMP-dependent protein kinase (Greengard, 1978, 1981; Nestler and Greengard 1983, 1984). Within the mammalian central nervous system (CNS), cyclic GMP-dependent protein kinase is highly enriched in cerebellar Purkinje cells (Schlichter et al., 1980; Lohmann et al., 1981; De Camilli et al., 1984). A soluble 23,000-dalton substrate for cyclic GMP-dependent protein kinase (G-substrate) was found to be relatively enriched in cerebellum (Schlichter et al., 1978; Aswad and Greengard, 1981a, b), and preliminary evidence indicated that, within the cerebellum, this protein was enriched in Purkinje cells (Schlichter et al., 1980). Recently, serum antibodies have been prepared against G-substrate (Nairn et al., 1982).

Using immunoprecipitation, radioimmunoassay (RIA), and peptide-mapping techniques, we have now carried out a detailed investigation of the regional and cellular distribution of G-substrate in the mammalian CNS. The results obtained indicate that G-substrate is highly concentrated in cerebellum, where it is specifically localized to Purkinje cells.

Materials and Methods

Materials. ATP, cyclic GMP, EDTA, HEPES, Tris, and phenylmethylsulfonyl fluoride (PMSF) were from Sigma Chemical Co. (St. Louis, MO). Thermolysin and Pansorbin were from Calbiochem-Behring Corp. (San Diego, CA), Nonidet P-40 was from Particle Data Laboratories (Elmhurst, IL), and 3-isobutyl-1-methylxanthine was from Aldrich Chemical Co., (Milwaukee, WI). Thin-layer cellulose (TLC) sheets (type 13255) were from Eastman Kodak (Rochester, NY), and Sephadex G-25 was from Pharmacia Fine Chemicals (Piscataway, NJ). All other chemicals were reagent grade. [γ -³²P]ATP was synthesized according to the method of Glynn and Chappell (1964).

Purification of G-substrate and phosphatase inhibitor-1. G-Substrate was purified from rabbit cerebella through step 6 (Aswad and Greengard, 1981a). Phosphatase inhibitor-1 was purified from rabbit skeletal muscle essentially by the method of Nimmo and Cohen (1978), except that an additional ion-exchange chromatography step was performed using CM-cellulose (Aswad and Greengard, 1981a) and gel filtration was performed using Ultrogel AcA44 (LKB Instruments, Inc., Gaithersburg, MD).

Preparation of rabbit tissue extracts. Preparation of total cytosol and of pH 2/6 acid extracts from rabbit cerebellum, cortex, hippocampus, and caudate was carried out using the method of Aswad and Greengard (1981a), except that in some experiments the acid extract samples were

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concentrated 5-fold. For this purpose, the final pH 2/6 extract was dialyzed against 25 mM NH_4HCO_3 /5 mM 2-mercaptoethanol, lyophilized to dryness, redissolved in the required volume of 25 mM Tris-HCl (pH 7.6)/1 mM EDTA/15 mM 2-mercaptoethanol, and dialyzed against the same buffer. This procedure had no effect on the subsequent phosphorylation pattern.

Preparation of tissue extracts from mutant mice. Neurological mutant mice C57Bl/6J Purkinje cell degeneration (PCD; *pcd/pcd*), C3HeB/FeJ nervous (*nr/nr*), and C57BL/6 $\times$ CB-A^{wi}/A weaver (*wv/wv*), and their respective age-matched wild-type controls were obtained from Jackson Laboratories (Bar Harbor, ME). PCD and nervous mutants selectively lose greater than 90% of cerebellar Purkinje cells by the age of 2 months, with no significant loss of other cell types (Landis, 1973; Landis and Mullen, 1978). PCD and nervous are autosomal recessive mutations; thus, heterozygous or wild-type controls are phenotypically normal (Landis, 1973; Landis and Mullen, 1978). In weaver mutants, granule cells are essentially absent by the age of 3 weeks (Rakic and Sidman, 1973a; Roffler-Tarlov and Sidman, 1978), with no significant loss of other cell types (Rakic and Sidman, 1973b). Heterozygous weavers lose a small fraction of cerebellar granule cells but are behaviorally normal (Roffler-Tarlov and Sidman, 1978).

PCD, nervous, and weaver mice were sacrificed at 3.5 to 4 months of age by cervical dislocation. Their cerebella were rapidly dissected and immediately placed into ice-cold "homogenization buffer" (10 mM HEPES (pH 7.4), 1 mM EDTA, 30 mM 2-mercaptoethanol, 100 μM PMSF, 0.1% (v/v) isopropanol) containing 0.32 M sucrose. All subsequent steps were carried out at 4°C. Average cerebellar weights were: PCD, 33.3 mg; PCD controls, 64.3 mg; nervous, 40.2 mg; nervous controls, 64.3 mg; weaver, 25.8 mg; and weaver controls, 51.5 mg. Cerebella from control mice were homogenized individually and cerebella from mutant mice were homogenized in pairs, in 5:1 (v/w) "homogenization buffer." Homogenates were centrifuged at $27,000 \times g$ for 20 min. Aliquots (0.25 ml) of the supernatants were desalted on a 2.5-ml bed of Sephadex G-25 in a 3-ml syringe using a modification of the centrifuge desalting technique (Helmerhorst and Stokes, 1980). The eluants were then boiled for 2 min to destroy the activities of endogenous kinases and phosphatases and then were used as the tissue extracts. G-Substrate (Aswad and Greengard, 1981a) and phosphatase inhibitor-1 (Nimmo and Cohen, 1978) are stable to such heat treatment.

Phosphorylation of 23,000-dalton phosphoprotein. The preparation of rabbit and mouse extracts, as described above, results in complete dephosphorylation of G-substrate (D. J. Schlichter, unpublished results). Phosphorylation was carried out in (final concentrations): 10 mM HEPES (pH 7.4), 0.3 mM EDTA, 1 mM 3-isobutyl-1-methylxanthine, 1 μM cyclic GMP, 10 mM MgCl_2 , 4 $\mu\text{g}/\text{ml}$ of cyclic GMP-dependent protein kinase (purified by the method of Walter et al., 1980), the indicated concentration of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, and appropriate volumes of rabbit or mouse tissue extract. Purified G-substrate and phosphatase inhibitor-1 (1 to 2 $\mu\text{g}/100 \mu\text{l}$) were used as standards. (Phosphatase inhibitor-1, a substrate for cyclic AMP-dependent protein kinase, has been shown to be phosphorylated at the same site by cyclic GMP-dependent protein kinase *in vitro* (Hemmings et al., 1984).) The reaction was initiated by the addition of ATP and carried out for 45 min at 30°C. Maximal phosphorylation of G-substrate, of phosphatase inhibitor-1, and of the 23,000-dalton phosphoprotein from various brain regions was obtained using these incubation conditions (D. J. Schlichter and A. C. Nairn, unpublished observations).

For peptide-mapping experiments, using rabbit brain acid extracts, 500 to 600 μg of protein were phosphorylated in a final volume of 1 ml with 2 to 5 μM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (7 to 8×10^7 cpm/nmol). The reaction was stopped with an SDS-containing stop solution (Casnellie et al., 1978), and 150- μl aliquots were run on 12% polyacrylamide gels. The gels were dried and autoradiography was performed as described (Casnellie et al., 1978). The 23,000-dalton phosphoprotein band was located from the autoradiogram and excised from the gel, and peptide mapping was performed as described below. Phosphatase inhibitor-1 is present in much lower concentration than is G-substrate in cerebellum. Therefore, for the quantitative determination of the amount of phosphatase inhibitor-1 in cerebellum (see Table I), a modification of the standard procedure was used. Specifically, prolonged electrophoresis was performed such that the dye front had run off the gel the equivalent of 4 cm. Under these conditions, the 23,000-dalton phosphoprotein band, from all brain regions studied, separated into two bands, the upper of which co-migrated with purified G-substrate and the lower of which co-migrated with purified phosphatase inhibitor-1. These distinct bands were subjected to peptide mapping as described below.

For immunoprecipitation experiments using cytosol from rabbit or mouse brain, equal amounts of protein from each sample (20 to 100 μg) were phosphorylated, under the conditions described above, in a final volume of 50 μl with 10 μM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (4 to 5×10^6 cpm/nmol). The reactions were stopped by the addition of an EDTA-containing "stop" solution (final concentrations, 15 mM EDTA/150 mM NaCl/50 mM NaF/50 mM Tris-HCl (pH 7.4)/0.02% sodium azide/1% Nonidet P-40), followed by boiling for 30 sec, and the resulting samples were used for immunoprecipitation as described below. The reactions of paired samples were stopped by the addition of SDS-containing stop solution, and the resulting samples were used for analysis of total phosphorylated proteins.

Proteolytic digestion of the 23,000-dalton phosphoprotein with thermolysin. The 23,000-dalton phosphoprotein band was excised as described above, washed with 3×10 ml of isopropanol/acetic acid (10%/10%), and the paper residue was removed. The gel piece was then washed with 2×10 ml of methanol (50%), lyophilized, reswollen into 1 ml of 50 mM NH_4HCO_3 containing 0.3 mg/ml of thermolysin, and digested at 37°C for 20 hr (in a 1.5-ml Microfuge tube). The gel piece was removed from the tube and washed (at 37°C) in an additional 0.5 ml of 50 mM NH_4HCO_3 , the wash and digest were combined and centrifuged to remove particulate material, and the supernatant was lyophilized. (Approximately 90 to 95% of all counts were removed from the gel piece by this method. Inclusion of a trace of phenol red in the first digestion buffer helped to ensure both that the pH was correct for digestion and that the gel piece was washed adequately.) The final dried sample was counted in the Microfuge tube by Cerenkov counting, and a volume of pyridine/acetic acid/ H_2O (1%/10%/89%) (pH 3.5) proportional to the amount of radioactivity was added to yield equal (about 500 to 1000) counts per minute per microliter. Replicate samples for each brain region were routinely pooled in a 1.5-ml Microfuge tube and centrifuged for 2 min to remove any residual debris. Aliquots (1 to 10 μl) were spotted on 20×20 cm TLC plates (Eastman) in the bottom right corner. Electrophoresis was performed at 400 V until the basic fuchsin dye spotted along with the sample had migrated 5 to 6 cm. The plates were dried, and ascending chromatography in pyridine/butanol/acetic acid/ H_2O (15/10/3/12) was performed twice. The plates were dried and autoradiography was performed. Radioactivity in specific peptides was determined by excising the part of the plate containing the peptide and counting by liquid scintillation spectrophotometry. Co-migration experiments were performed by mixing aliquots of digests from gel pieces containing pure G-substrate or phosphatase inhibitor-1 with aliquots of digests of gel bands containing the 23,000-dalton phosphoprotein band from different brain regions.

Calculation of the relative molar concentrations of G-substrate and phosphatase inhibitor-1 in each brain region took into account the fact that G-substrate is phosphorylated at two different sites (Aitken et al., 1981), whereas phosphatase inhibitor-1 is phosphorylated at only one site (Cohen et al., 1977). For each brain region, the fraction of the total counts applied to the TLC plate which were recovered, in either the two G-substrate phosphopeptides or the one phosphatase inhibitor-1 phosphopeptide, along with the total concentration of 23,000-dalton phosphoprotein found in the acid extract, was used to calculate the concentration of G-substrate and phosphatase inhibitor-1.

Immunoprecipitation of phosphorylated G-substrate. Phosphorylated G-substrate was immunoprecipitated by a modification of the Protein A-bearing *Staphylococcus* (SAC)-immunoprecipitation technique (Ivarie and Jones, 1979). Phosphorylated samples were precleared by incubation with 20 μl of a 10% suspension of SAC (Pansorbin) in "NET" buffer (containing 150 mM NaCl, 15 mM EDTA, 50 mM Tris (pH 7.4), 0.02% sodium azide, 50 mM NaF, and 1% Nonidet P-40) for 15 min on ice followed by centrifugation for 4 min in a Beckman Microfuge to obtain the precleared supernatants. These samples were incubated for 30 to 60 min on ice with 10 μl of immune serum specific for the phosphorylated form of G-substrate (Nairn et al., 1982). (Ten microliters of immune serum was sufficient to precipitate 4 pmol of G-substrate. In all immunoprecipitation experiments, at least a 20-fold excess of immune serum was added.) Thirty microliters of the 10% SAC suspension were added to each sample and incubation was carried out for an additional 15 min. The samples were then centrifuged for 4 min in a Microfuge, the supernatants were aspirated, and the pellets, containing the antibody-antigen complex, were washed in NET buffer and centrifuged again; then the supernatants were aspirated. The resulting pellets were resuspended in SDS-containing stop solution, followed by boiling, and aliquots were subjected to SDS-polyacrylamide gel electrophoresis. The gels were dried and autoradiography was per-

formed. Bands corresponding to immunoprecipitated phospho-G-substrate were cut from the dried gel and counted by liquid scintillation spectrometry. Under optimum conditions, the immunoprecipitation assay precipitated 75% of phospho-G-substrate present in the phosphorylated extract, as determined in control experiments using purified [32 P]-G-substrate of known concentration. The exact percentage of phospho-G-substrate precipitated varied slightly among experiments, but within any given set of assays a highly reproducible percentage of phospho-G-substrate was precipitated over a broad range of G-substrate concentrations.

RIA for G-substrate. Antibodies specific for either dephospho-G-substrate or phospho-G-substrate were prepared and RIA was performed, as described (Nairn et al., 1982).

Protein determinations. Protein determinations were carried out by the method of Lowry et al. (1951), following precipitation of the samples with trichloroacetic acid, using bovine serum albumin fraction V as standard.

Results

Regional distribution of G-substrate in mammalian brain studied by immunoprecipitation and RIA. Figure 1 shows an autoradiogram of an SDS-polyacrylamide gel in which phosphorylated G-substrate was immunoprecipitated from rabbit cerebellar cytosol using phospho-G-substrate-selective antibodies. No G-substrate could be detected by this procedure in samples of cytosol obtained from rabbit cortex (Fig. 1), hippocampus, or caudate (data not shown).

The concentration of G-substrate in various rabbit brain regions was measured quantitatively using an RIA sensitive for G-substrate (Nairn et al., 1982). Since the conditions used to prepare tissue extracts resulted in the complete dephosphorylation of G-substrate, an RIA employing dephospho-selective antibodies was used (Nairn et al., 1982). (Similar results were obtained if samples were first phosphorylated by the addition of cyclic GMP-dependent protein kinase, cyclic GMP, and ATP, and then analyzed by RIA using phospho-G-substrate-selective antibodies (data not shown).) The concentration of G-substrate measured by RIA in total homogenates from cerebellum was 10.3 ± 1.87 pmol/mg (mean $\pm$ SD, $n = 8$). Following centrifugation at $100,000 \times g$ for 30 min, G-substrate was enriched (27.2 ± 6.5 pmol/mg (mean $\pm$ SD, $n = 6$)) in the cytosol fraction. The apparent concentrations of G-substrate measured by RIA in comparable cytosol fractions prepared from cortex, hippocampus, and caudate were 0.26 ± 0.11 (mean $\pm$ SD, $n = 5$), 0.34 ± 0.10 ($n = 3$), and 0.64 ($n = 1$), respectively. These values represent levels of G-substrate in noncerebellar brain regions approximately 1 to 2% of that measured in cerebellum. The concentration of G-substrate in noncerebellar brain regions was measured only in cytosol samples because addition of high concentrations of homogenate interfered with the RIA.

Regional distribution of G-substrate and phosphatase inhibi-

Figure 1. Immunoprecipitation of G-substrate from rabbit brain cytosol. Samples of cytosol from cerebellum or cortex were phosphorylated by the addition of cyclic GMP-dependent kinase and [γ - 32 P]ATP. Immunoprecipitation was performed using phospho-G-substrate-specific antibodies. Following immunoprecipitation, samples were subjected to SDS-PAGE and phosphoprotein bands were visualized by autoradiography. Autoradiograms were exposed for equal times. The *left* lane in each pair shows the pattern of the total 32 P-labeled cytosol, and the *right* lane shows the pattern of the pellet obtained following immunoprecipitation. Longer exposure of the cortex immunoprecipitation sample did not reveal any 23,000-dalton phosphoprotein.

tor-1 in mammalian brain studied by peptide mapping. In previous studies, a 23,000-dalton phosphoprotein band was found in acid extracts from all brain regions examined (Aswad and Greengard, 1981a). In the present study, the average amount of the 23,000-dalton phosphoprotein band in noncerebellar brain regions was 35 to 45% of that in the cerebellum. The results from immunoprecipitation and RIA described above indicated that, in mammalian brain, G-substrate was highly enriched in cerebellum. These results suggested that the 23,000-dalton phosphoprotein band found in acid extracts from non-cerebellar brain regions was not predominantly G-substrate. We have therefore investigated the identity of the 23,000-dalton phosphoprotein band in acid extracts from various brain regions using peptide-mapping techniques. In these studies, samples from the different brain regions were phosphorylated by cyclic GMP-dependent protein kinase and separated by SDS-PAGE. The phosphoproteins of interest were located by autoradiography and subjected to exhaustive proteolytic digestion

with thermolysin, and the peptides were analyzed by two-dimensional peptide mapping.

Figure 2A shows the peptide map obtained from standard G-substrate. Two major phosphopeptides (G1 and G2) were obtained with electrophoretic and chromatographic mobilities corresponding to those expected from the known phosphopeptide sequences (Aitken et al., 1981). A similar peptide map (Fig. 2C) was generated from the 23,000-dalton cerebellar phosphoprotein. Different and more complex maps were obtained for cortex, hippocampus, and caudate. The major phosphopeptide obtained from cortex (Fig. 2D, *single arrow*), hippocampus, and caudate (data not shown) co-migrated with the major phosphopeptide (I1) obtained from standard phosphatase inhibitor-1 (Fig. 2B). In the cortex, hippocampus, and caudate, several minor phosphopeptides were also detected, two of which (see Fig. 2D, *double arrows*) co-migrated with the two phosphopeptides obtained from purified G-substrate.

The relative concentrations of G-substrate and phosphatase

Figure 2. Autoradiographs showing phosphopeptides obtained following thermolytic digestion of G-substrate, phosphatase inhibitor-1, and the 23,000-dalton phosphoprotein band from rabbit cerebellum and cortex. Purified G-substrate and phosphatase inhibitor-1, and samples of acid extract prepared from rabbit cerebellum and cortex, were phosphorylated by the addition of cyclic GMP-dependent protein kinase and [γ - 32 P]ATP. Samples were subjected to SDS-PAGE and phosphoproteins were visualized by autoradiography. The regions of the gel corresponding to G-substrate, phosphatase inhibitor-1, and the 23,000-dalton phosphoprotein band from cerebellum and cortex were excised and subjected to exhaustive digestion using thermolysin. Digests were applied to cellulose TLC plates (origin, o, at bottom right corner) and were separated, first by electrophoresis in the horizontal dimension (negative pole left), and then by ascending chromatography in the vertical dimension. A, G1 and G2 are the two phosphopeptides obtained from digestion of G-substrate. B, I1 is the one phosphopeptide obtained from digestion of phosphatase inhibitor-1. C, The *double arrows* indicate the two phosphopeptides obtained from digestion of the 23,000-dalton (23K) phosphoprotein band in the cerebellar sample; these two phosphopeptides co-migrate with the two phosphopeptides obtained from pure G-substrate. D, The *single arrow* indicates the major phosphopeptide obtained from digestion of the 23,000-dalton phosphoprotein band in the cortex sample; this phosphopeptide co-migrates with that obtained from pure phosphatase inhibitor-1. The *double arrows* indicate the two phosphopeptides in the cortex which co-migrate with those obtained from pure G-substrate.

inhibitor-1 in each brain region studied were calculated from the peptide-mapping data and the results are summarized in Table I. The concentration of G-substrate in cerebellum was arbitrarily set at 100 and the other data were expressed as a percentage of this number. The concentration of G-substrate found in cortex, hippocampus, and caudate was 4 to 5% of that measured in cerebellum. The concentration of phosphatase inhibitor-1 in cerebellum was about 4% of the concentration of G-substrate in this brain region. The concentration of phosphatase inhibitor-1 was 5- to 10-fold higher in cortex, hippocampus, and caudate than in cerebellum.

These data indicated that the 23,000-dalton phosphoprotein band present in acid extracts from cerebellum consisted almost exclusively of G-substrate. In contrast, the phosphoprotein band of similar molecular weight present in acid extracts from other brain regions consisted of several phosphoproteins, the predominant one being phosphatase inhibitor-1. The concentrations of G-substrate in noncerebellar brain regions relative to that in cerebellum were higher when calculated from peptide-mapping data (Table 1) than when measured by RIA (see previous section). It is likely that the RIA more accurately measured the very low concentrations of G-substrate in noncerebellar brain regions due to the greater sensitivity of this technique. The peptide-mapping technique does appear, however, to give qualitatively correct results: qualitatively similar distributions of G-substrate were found by peptide mapping and by RIA (this study); qualitatively similar distributions of phosphatase inhibitor-1 were found by peptide mapping (this study) and by rocket immunoelectrophoresis (S. Shenolikar and S. J. Strada, personal communication).

Phosphoamino acid analysis. Purified G-substrate and phosphatase inhibitor-1 are phosphorylated on phosphothreonine (Aitken et al., 1981). Phosphoamino acid analysis indicated that the two major peptides derived from the 23,000-dalton cerebellar phosphoprotein contained only phosphothreonine (data not shown). Similarly, the major peptide derived from the 23,000-dalton phosphoprotein present in extracts from cortex, hippocampus, and caudate contained only phosphothreonine. These results are consistent with the conclusion that the major peptides observed in the peptide-mapping studies were derived from G-substrate and phosphatase-inhibitor-1.

Cellular distribution of G-substrate in cerebellum. We have examined the distribution of G-substrate in cerebella from mutant mice deficient in either Purkinje cells (PCD and nervous) or granule cells (weaver) using both the immunoprecipitation and RIA techniques described above. Figure 3 shows an autoradiogram of an SDS-polyacrylamide gel in which G-substrate was immunoprecipitated from phosphorylated samples of cerebellar cytosol prepared from these various strains of

mice. A phosphoprotein band was obtained from normal mouse cytosol (Fig. 3, lane 1) which co-migrated with the 23,000-dalton G-substrate from rabbit on SDS-PAGE. (The levels of G-substrate were lower in mouse than in rabbit.) G-substrate has also been detected in cerebella from the rat, guinea pig, cow, chicken, and humans using this procedure (data not shown).

The concentration of G-substrate in cerebella from both PCD and nervous mutant mice (Fig. 3, lanes 2 and 3) was markedly reduced in comparison to the normal age-matched, wild-type control (Fig. 3, lane 1). The band representing phospho-G-substrate was faintly visible in the nervous mutant (Fig. 3, lane 3) and essentially absent in the PCD mutant (Fig. 3, lane 2). In contrast, the concentration of G-substrate in the Weaver mutant (Fig. 3, lane 4) was high compared to the normal age-matched control (Fig. 3, lane 1). The reduced concentration of G-substrate in the PCD and nervous mutants did not appear to be due to the presence of inhibitory elements in the mutant extracts since the expected levels of G-substrate were observed when cerebellar cytosols from normal and mutant mice were mixed. In agreement with the immunoprecipitation experiments (Fig. 3), the concentration of G-substrate measured by RIA showed a significant reduction in both PCD and nervous mutants when compared to controls (Table II). The concentration of G-substrate in PCD mutants was 3.9% of that measured in the control animals whereas the concentration in nervous mutants was 9.2% of control values.

Discussion

The present studies indicate that G-substrate is highly enriched in the cerebellum. The enormous reduction in the concentration of G-substrate measured in cerebella from PCD and nervous mutant mice and the increase in its concentration in weaver mutant mice indicate that this protein is localized within Purkinje cells. This conclusion is supported by the increase in G-substrate concentration observed in cerebella of x-irradiated rats (Dolphin et al., 1983), since neonatal x-irradiation produces a 90 to 98% reduction in most types of cerebellar interneurons, resulting in a relative enrichment of Purkinje cells. Recently, direct evidence for a Purkinje cell localization has been obtained from immunocytochemical studies using G-substrate antibodies (P. Miller, C. C. Ouimet, and P. Greengard, unpublished results). Assuming that G-substrate in cerebellum is localized to Purkinje cells and that Purkinje cells constitute 10% of the volume of the cerebellum, we have estimated the concentration of G-substrate in Purkinje cells to be $10.8 \pm 1.44 \mu\text{M}$ (mean $\pm$ SD, $n = 8$).

The results also demonstrate that the 23,000-dalton phosphoprotein band found in acid extracts from noncerebellar brain regions (Aswad and Greengard 1981a), although containing some G-substrate, consists predominantly of phosphatase inhibitor-1. In contrast to the results obtained with G-substrate, the concentration of phosphatase inhibitor-1 was significantly lower in cerebellum than in other major brain regions. Phosphatase inhibitor-1, a physiological substrate for cyclic AMP-dependent protein kinase, has been purified from rabbit skeletal muscle and shown to be a potent inhibitor of phosphatase-1 (Nimmo and Cohen, 1978). Phosphatase inhibitor-1 has several properties in common with G-substrate. In addition to similar molecular weights, both are phosphorylated on threonine residues, are heat stable and acid soluble, and have similar amino acid sequences around their phosphorylation sites (Aitken et al., 1981). Recently, it has been shown that the phosphorylated form of G-substrate can act as a phosphatase inhibitor (P. Simonelli, H. -C. Li, A. C. Nairn, and P. Greengard, manuscript in preparation). The present results suggest a specific role for G-substrate in Purkinje cells analo-

TABLE I

The relative molar concentrations of G-substrate and phosphatase inhibitor-1 present in the 23,000-dalton phosphoprotein band from various brain regions

The relative molar concentrations (RMC) of G-substrate and phosphatase inhibitor-1 in each brain region were calculated from the peptide-mapping data as indicated in the text. Peptide-mapping experiments were performed six times, and qualitatively similar results were obtained in all experiments. The results shown represent the average of data obtained from two of these experiments.

Brain Region	G-Substrate	Phosphatase Inhibitor-1
	RMC	
Cerebellum	100	3.6
Cortex	5	19
Hippocampus	4	36
Caudate	5	28

Figure 3. Immunoprecipitation of G-substrate from cytosol of cerebella from normal and neurological mutant mice. Experiments were carried out as described in the legend to Figure 1. Autoradiograms were exposed for equal times. Longer exposure of the autoradiogram of the nervous mutant mouse sample revealed a distinct band corresponding to G-substrate in this mutant (data not shown).

TABLE II

Concentration of G-substrate in cerebella from PCD and nervous mutant mice

G-Substrate concentrations were determined by RIA as described (Nairn et al., 1982). The concentration of G-substrate represents the mean $\pm$ SD for three control mice or three pairs of mutant mice.

Mutation	Genotype	G-Substrate <i>fmol/mg of cytosol protein</i>
PCD	+/?	1510 $\pm$ 302
	PCD/PCD	58.3 $\pm$ 10.4
Nervous	+/?	1580 $\pm$ 145
	NR/NR	145 $\pm$ 73.6

gous to that played by phosphatase inhibitor-1 in many other types of cells.

The distribution of G-substrate closely parallels that of cyclic GMP-dependent protein kinase (Schlichter et al., 1980; Lohman et al., 1981). Furthermore, cerebellar guanylate cyclase (Ariano et al., 1982) is primarily located in Purkinje cells. The localization of several elements of the cyclic GMP-dependent protein phosphorylation system in the Purkinje cell suggests an important and selective role for cyclic GMP-dependent protein phosphorylation in this type of neuron.

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