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Immunohistochemical localization of cyclic GMP-dependent protein kinase in mammalian brain

(Purkinje cell/protein phosphorylation/blood vessels/smooth muscle cells)

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ABSTRACT **The distribution of cyclic GMP-dependent protein kinase in rat brain has been studied by an immunological approach involving radioimmunoassay and fluorescence immunohistochemistry. Data obtained by radioimmunoassay indicate that cyclic GMP-dependent protein kinase is 20- to 40-fold more concentrated in cerebellum than in other brain regions. Immunohistochemical experiments demonstrate that the high concentration of immunoreactivity of the protein kinase in cerebellum is attributable to Purkinje cells. Immunoreactivity in these cells is homogeneously distributed throughout the cell (perikarya, dendrites, and axons) with the exception of the nucleus. No other neurons either in the cerebellum or in other brain regions were stained by antiserum to the protein kinase. Immunoreactivity, however, was found throughout the brain on smooth muscle cells of blood vessels.**

Several studies suggest that cyclic GMP (cGMP) may play an important role in the function of the nervous system (for reviews, see refs. 1–6). In contrast to cyclic AMP (cAMP), which is rather homogeneously distributed in different areas of the brain, cGMP is more specifically localized and it is 10–50 times more concentrated in cerebellum than in other brain regions (1, 4, 7–9). cGMP is thought to exert its effects through a cGMP-dependent protein kinase (cGK) (6). cGK activity has been detected in brain, especially in cerebellum, which was found to have one of the highest levels of cGK activity of the mammalian body (10–13). In the cerebellum a specific endogenous substrate for cGK has also been identified, purified, and characterized (14–17). Biochemical studies have suggested that the high levels of cGMP (7–9) and cGK (13, 18) found in cerebellum are primarily due to Purkinje cells. In order to obtain more direct information on the localization of cGK in mammalian brain, we have carried out immunochemical and immunohistochemical studies. Some initial immunohistochemical studies (unpublished; carried out in collaboration with Floyd E. Bloom and Elena Battenberg of The Salk Institute), using an immunoperoxidase technique, gave results similar to those reported here using an immunofluorescence technique.

MATERIALS AND METHODS

Materials. Paraformaldehyde was purchased from Fisher, goat IgG antirabbit IgG from Miles, IgGsorb from The Enzyme Center (Boston, MA), and rhodamine-conjugated goat IgG antirabbit IgG from Cappel Laboratories (Cochranville, PA).

Preparation and Characterization of Anti-cGK Antiserum. cGK from bovine lung was purified to homogeneity by affinity chromatography on 8-(6-aminohexylamino)-cAMP-Sepharose and a specific antiserum against this enzyme was raised in rabbits as described (19). The specificity of the antiserum was tested by several criteria: (*i*) a single band of precipitation was observed when the antiserum was tested against a lung extract by the agar double-diffusion test; (*ii*) a single band, with the same electrophoretic mobility as that of pure cGK, was labeled when $NaDodSO_4$ gels of total cerebellum homogenates were radioimmunolabeled (20) with anti-cGK antiserum followed by ^{125}I-labeled protein A; and (*iii*) the antiserum quantitatively precipitated the cGK labeled with ^{32}P-labeled 8-azidoinosine 3′,5′-cyclic monophosphate (8-N_3-[^{32}P]cIMP) from rat cerebellum but did not precipitate the 8-N_3-[^{32}P]cAMP-labeled regulatory subunits, R_I and R_{II}, of cAMP-dependent protein kinase (Fig. 1) nor did it precipitate any other endogenously phosphorylated protein from rat cerebellum. It was particularly important to establish that our antiserum did not recognize R_I and R_{II} because (*i*) R_I and R_{II} were the most likely contaminants in the preparations of purified cGK used for immunization and (*ii*) it has been proposed (21, 22) that cAMP- and cGMP-dependent protein kinases are homologous enzymes with similar structures.

Radioimmunoassay (RIA) of cGK. RIA determinations of cGK levels in various brain regions were performed with extracts prepared as for experiments of 8-N_3-[^{32}P]cIMP incorporation (19). Preliminary experiments carried out by 8-N_3-[^{32}P]cIMP labeling showed that ≈90% of the cerebellar cGK was recovered in such extracts. Details of the RIA procedure are reported elsewhere (23).

Immunohistochemistry. Rats were anesthetized by ether and transcardially perfused at a pressure of ≈120 mm of Hg (1 mm Hg = 133 Pa) for 5 min with ice-cold 120 mM sodium phosphate buffer (pH 7.4) and then for 10–15 min with ice-cold 4% (wt/vol) formaldehyde (freshly prepared from paraformaldehyde) in the same buffer. Brains were subsequently removed and sliced into 1- to 3-mm-thick coronal or sagittal slabs, which were immersed in the same ice-cold fixative for an additional 3 hr. The slabs were then washed several times in phosphate-buffered saline (pH 7.4), passed through a series of increasing concentrations (wt/vol) of sucrose (12%, 15%, and 18%) in phosphate-buffered saline, and stored in 18% sucrose/0.03% NaN_3 in phosphate-buffered saline until sectioned. For sectioning, tissue slabs were frozen either on dry ice or by immersion in isopentane chilled with liquid nitrogen, equilibrated in a cryostat at −25°C, sectioned serially at thicknesses of 6–8 μm, mounted on glass slides, and briefly air dried. In order to obtain a good adhesion of frozen sections to glass slides, the slides were

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Abbreviations: cAMP, adenosine 3′,5′-cyclic monophosphate; cGMP, guanosine 3′,5′-cyclic monophosphate; cGK, cGMP-dependent protein kinase; 8-N_3-cIMP, 8-azidoinosine 3′,5′-cyclic monophosphate; R_I and R_{II}, regulatory subunits of the type I and type II cAMP-dependent protein kinases, respectively; RIA, radioimmunoassay.

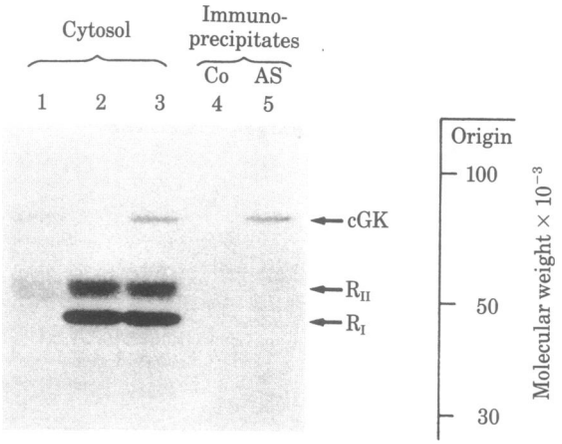

FIG. 1. Autoradiograph of $NaDodSO_4$/polyacrylamide gel showing that cGK is specifically precipitated by the anti-cGK antiserum in extracts of rat cerebellum. Lanes 1–3, cerebellar extracts photoaffinity labeled by 8-N_3-[^{32}P]cIMP prepared as described (19). In order to demonstrate the specificity of labeling of the cyclic nucleotide-binding proteins [cGK and regulatory subunits of type I (R_I) and type II (R_{II}) cAMP-dependent protein kinases], photoaffinity labeling was carried out under standard conditions in the absence of nonradioactive nucleotides (lane 3), in the presence of 1 μM cGMP (lane 2), and in the presence of 20 μM cAMP (lane 1). Proteins recognized by preimmune serum (Co) or immune serum (AS) in extracts identical to that applied to lane 3 were immunoprecipitated as described in ref. 19 and applied to lanes 4 and 5, respectively.

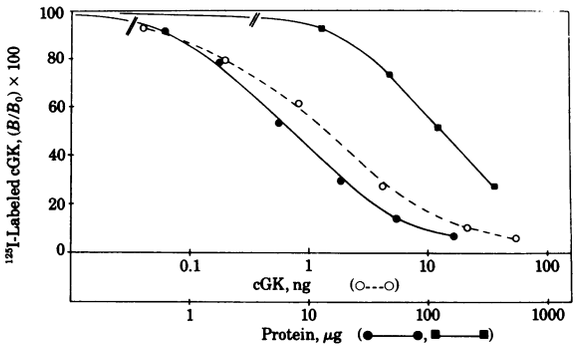

FIG. 2. RIA of cGK showing the competition (B/B_0) of cGK purified from bovine lung (○—○) or of endogenous cGK in extracts of rat cerebellum (●—●) or cerebral cortex (■—■) with bovine ^{125}I-labeled cGK for binding to antiserum against cGK. B is the ^{125}I-labeled cGK bound in the presence of various amounts of purified cGK or extracts from cerebellum and cerebral cortex; B_0 is the ^{125}I-labeled cGK bound in the absence of addition of unlabeled cGK. Each point represents the mean of closely agreeing triplicates.

pretreated by a brief immersion in a solution containing 1.5% (wt/vol) gelatin, 30% (vol/vol) ethanol, 0.1% chromium potassium sulfate, and 7% (vol/vol) acetic acid, followed by air drying. For immunostaining, sections were first incubated for 30 min in the presence of 0.1 M glycine buffered at pH 7.4 (Tris base), then for 3 hr at room temperature in the presence of the primary antiserum diluted in Triton buffer (0.3% Triton X-100/0.45 M NaCl/20 mM sodium phosphate buffer, pH 7.4) which also contained normal goat serum diluted 1:6. Subsequently, after several washings in Triton buffer, slices were incubated for 1 hr at 37°C in the presence of rhodamine-conjugated goat IgG anti-rabbit IgG diluted in Triton buffer containing normal goat serum diluted 1:6. Slices were then washed with Triton buffer, rinsed with phosphate-buffered saline, and finally mounted in 95% glycerol/5% phosphate-buffered saline and examined in a Zeiss Universal Photomicroscope II with epifluorescence optics. All results were replicated in at least four different animals. In some cases, after examination by fluorescence microscopy, sections were stained by toluidine blue and reexamined by bright-field microscopy.

RESULTS

Quantitative Estimation of cGK in Brain by RIA. cGK was detected by RIA in every region of the rat brain examined. However, the results obtained in four separate experiments indicated that the concentration (mean ± SEM) of this enzyme in the cerebellum (1.63 ± 0.30 pmol of cGK per mg of protein) is 20- to 40-fold higher than that in other brain regions. The results obtained for cerebellum and for cerebral cortex are shown in Fig. 2.

Immunohistochemistry. When frozen sections of several regions of the brain, including cerebellum (Fig. 3*a*), cerebral cortex, hippocampus, caudate nucleus, hypothalamus, thalamus, and various regions of the brain stem, were immunostained by using preimmune serum or anti-cGK serum preadsorbed with purified cGK as primary antiserum, only a barely detectable dark-red fluorescence (almost indistinguishable from the autofluorescence of the tissue) was observed. In contrast, when nonpreadsorbed anti-cGK immune serum was used as the primary antiserum, a bright red fluorescence was observed on the cerebellar cortex, the cerebellar white matter, the deep cerebellar nuclei, and, to a lesser extent, in some closely adjacent regions of the brain stem (Figs. 3 *b–f* and 4 *a–d*). The morphological distribution of immunoreactivity was consistent with a specific association with Purkinje cells. Immunoreactivity was present on Purkinje cell perikarya in the Purkinje cell layer. In other regions of the cerebellum it closely matched the expected structure (24–27) of Purkinje cell processes in those regions: dendrites in the molecular layer, axons in the granule cell layer and in the white matter, and nerve terminals in the deep cerebellar nuclei and in adjacent regions of the brainstem. Staining was homogeneously distributed throughout the cytoplasm of Purkinje cells but did not occur on the nucleus. In some cases, sections were stained with toluidine blue after examination of the pattern of immunofluorescent staining. A comparison of the two staining patterns of the same fields indicated that all Pur-

FIG. 3 (*on following page*). (*a–f*) Fluorescent localization of cGK immunoreactivity in rat cerebellum. Immunoreactive material appears white. (*a*) Cerebellar cortex after incubation with preimmune serum. M, molecular layer; G, granule cell layer. The faint fluorescence visible in this micrograph is due to background autofluorescence. (*b–f*) Sections of cerebellum after incubation with anti-cGK antiserum. Bright immunoreactivity is visible throughout the Purkinje cells with the exception of the nuclei. Dendrites are seen in the molecular layer M. (Dark nonfluorescent images visible in this layer are unstained cell bodies of nerve and glial cells as well as vessel lumena.) The structure of Purkinje cell dendrites is very different in sagittal (*b* and *e*) and coronal (*c* and *d*) sections, reflecting their unique arrangement in parallel planes that are sagittally oriented. The structure of the dendrites in coronal section of the molecular layer is particularly well illustrated in *d*. In *b*, immunoreactive axons of Purkinje cells are distinctly seen in the granule cell layer G (arrowheads) and in the white matter W. Thin arrows indicate the network of Purkinje cell recurrent axons. Interspersed with this network are bright dots, which are probably terminals of such axons. In *e*, the cGK immunoreactivity is shown to be specific for cerebellar Purkinje cells and cannot be observed in other brain regions; e.g., an adjacent portion of the brain stem (*). (*g*) Bright-field micrograph. After the photograph shown in *f* was taken, the section was stained with toluidine blue and the same field was rephotographed in bright-field. Comparison of the two figures shows that all Purkinje cells (as visualized by toluidine blue) are immunoreactive. (*a*, ×220; *b*, ×720; *c*, ×220; *d*, ×340; *e*, ×170; *f* and *g*, ×290.)

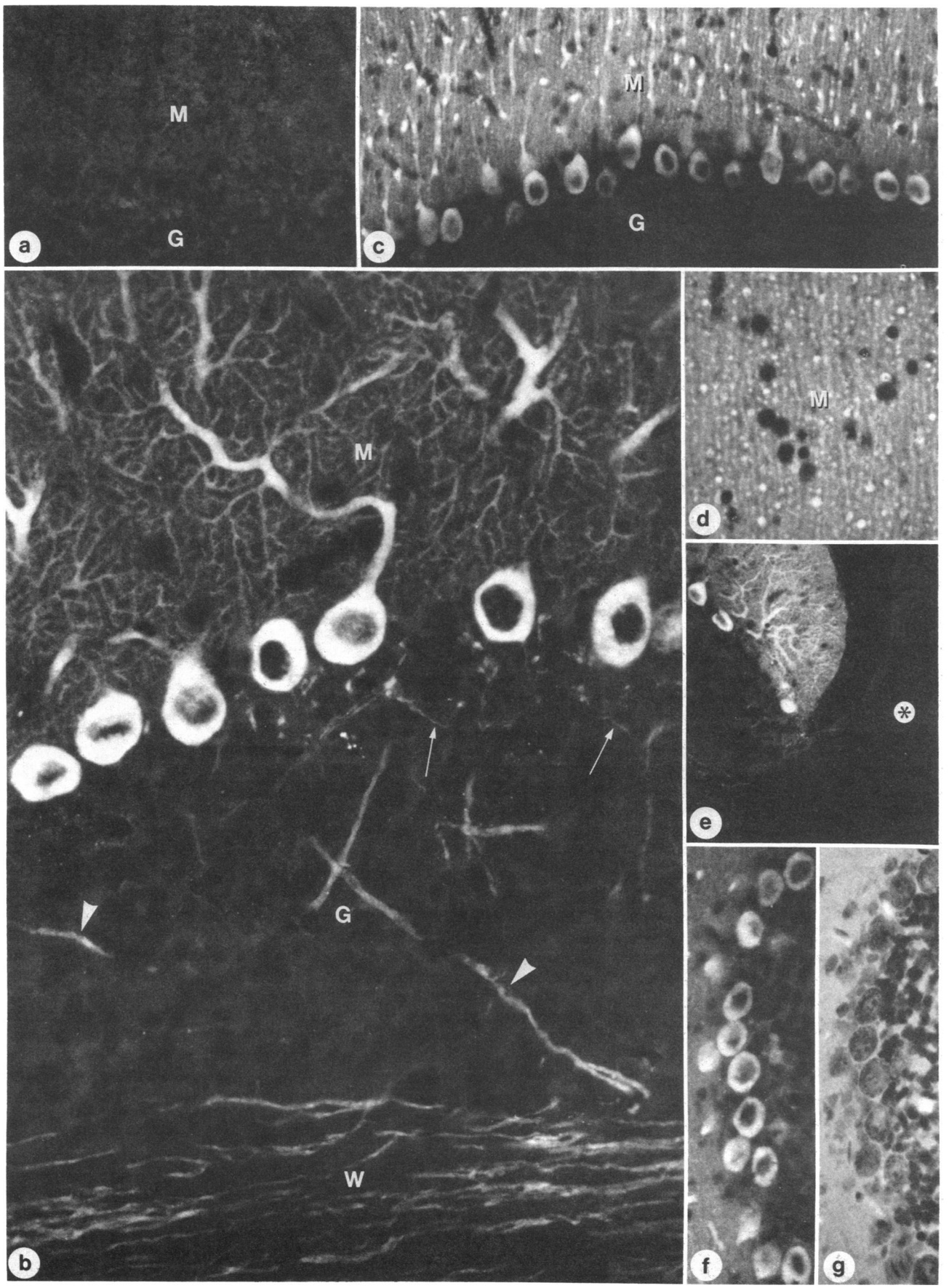

FIG. 3. (*Legend on preceding page.*)

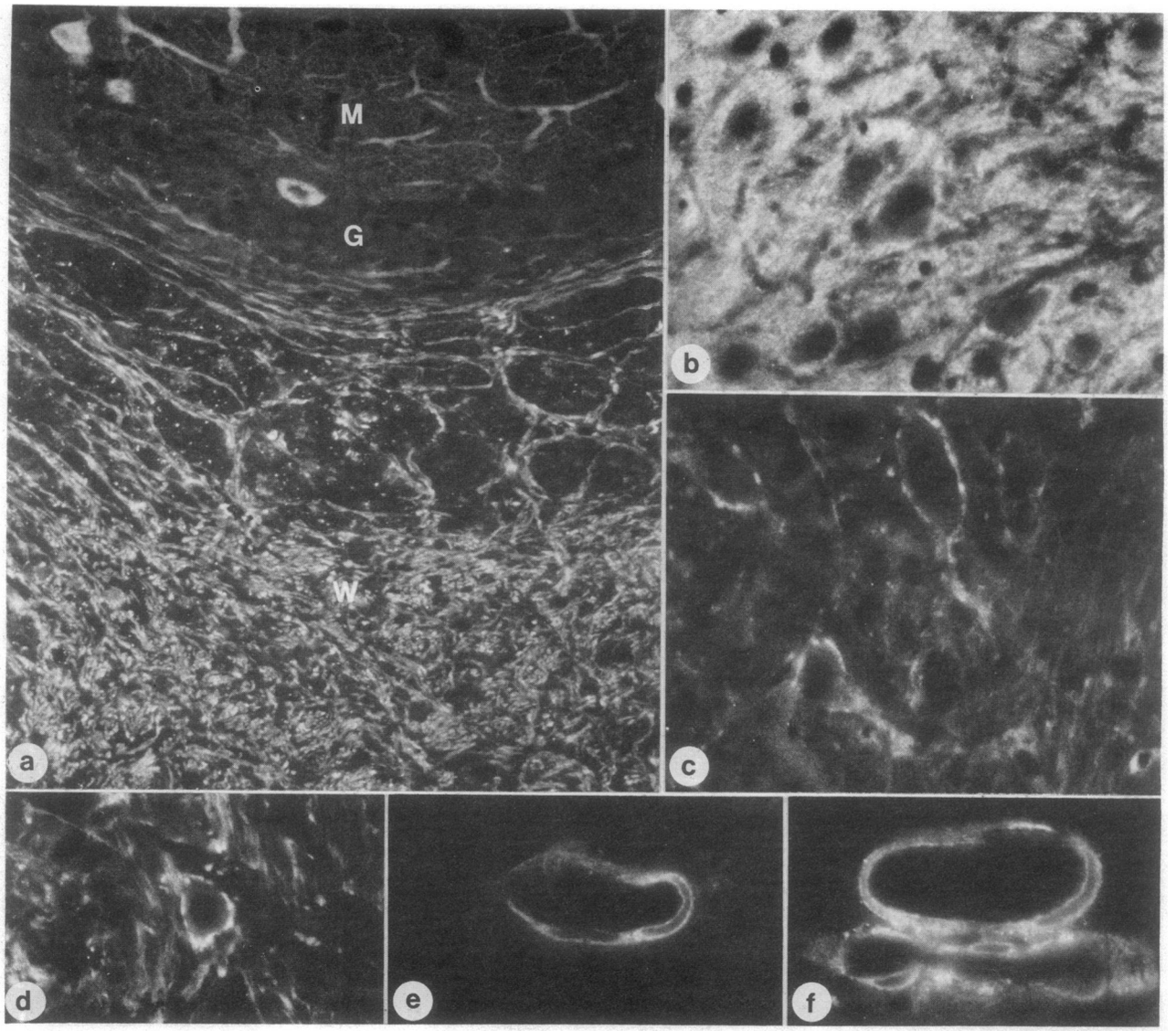

FIG. 4. Fluorescent localization of cGK immunoreactivity in cerebellum and blood vessels. (*a*) Portion of cerebellar cortex and of white matter immediately adjacent to the deep cerebellar nuclei. One can see immunoreactivity in the Purkinje cell bodies and dendrites as well as in their axons in the white matter W. The bottom of the photograph shows a portion of the white matter where Purkinje cell axons from several folia merge and intermesh before innervating the deep cerebellar nuclei. M, molecular layer; G, granule cell layer. (*b*) Neuron cell bodies of the deep cerebellar nuclei completely surrounded by immunoreactive axons originating from Purkinje cells and by their terminals. Note that the cell bodies are not stained. (*c* and *d*) Neurons in the vestibular complex surrounded by immunoreactive terminals and axons likely to be direct projections from Purkinje cells (see text). (*e* and *f*) Immunoreactivity in smooth muscle cells of blood vessels in cerebral cortex (*e*) and meningeal membranes (*f*). The cytoplasm, but not the nucleus, is immunostained. (*a* and *b*, ×295; *c*, ×340; *d*, ×285; *e* and *f*, ×315.)

kinje cells were immunostained (Fig. 3 *f* and *g*).

The arborization of the Purkinje cell dendrites in the molecular layer is clearly seen in Fig. 3 *b–e*. Because such arborization occurs in a two-dimensional array to form sheets sagitally oriented (24, 25), the pattern of immunoreactivity is very different in sagittal and in coronal sections. Dendritic branching is seen in sagittal sections (Fig. 3*b*). On the contrary, in coronal sections dendrites appear either as straight lines (if running in the plane of the section) or as discs (if cross-sectioned) (Fig. 3 *c* and *d*).

Axons are immunostained throughout their entire length, as shown in Figs. 3*b* and 4 *a–d*. They can be seen emerging from the Purkinje cell bodies, traversing the granule cell layer, merging into bundles in the white matter of the cerebellum, and penetrating the deep cerebellar nuclei. The recurrent axons that form a network aligned parallel to the Purkinje cell layer in the outermost portion of the granule cell layer (24–26) can also be identified. Furthermore, some axons could be observed emerging from the cerebellum and extending for short distances into the brainstem, where direct projections of Purkinje cells to the vestibular complex have been described (27).

The patterns of immunoreactivity at target sites of Purkinje cell innervation are shown in Fig. 4 *b–d*. In the deep cerebellar nuclei (Fig. 4*b*), which are the major targets of such innervation (24–27), immunoreactive material completely surrounds neuronal cell bodies, which are unstained. This pattern is consistent with the presence, around those neurons, of Purkinje cell terminals and of bundles of Purkinje cell axons that provide those terminals. The dot-like appearance of Purkinje cell terminals along the surface of target cells is better seen around neurons of the vestibular complex (Fig. 4 *c* and *d*), which is only sparsely innervated by Purkinje cells (27). Images suggestive of labeled

terminals can also be seen in the granule cell layer (Fig. 3*b*) at regions where terminals of recurrent Purkinje cell axons are known to occur (24–27).

The immunoreactivity on Purkinje cells was the only immunoreactivity detectable by the anti-cGK antiserum on neuronal elements in either the cerebellum or other regions of the brain. It was observed, however, that cells of another type were labeled by the anti-cGK antiserum. These were the smooth muscle cells of the cerebral vasculature (Fig. 4 *e* and *f*). Staining of these cells was, in general, less pronounced than staining of Purkinje cells and, in some experiments, it was hardly detectable. This was the case in the experiments in which we obtained the best photographs of Purkinje cells (Figs. 3 and 4 *a–d*). Staining of smooth muscle cells was observed both on the intrinsic vessels of the brain and on meningeal vessels. Immunoreactivity was localized in the cytoplasm of the smooth muscle cells and was not present on the nucleus. Staining by anti-cGK antiserum seems to be a general property of smooth muscle cells, occurring both in vessels and in other tissues that we have investigated (23).

DISCUSSION

Results reported in this paper confirm by an immunological approach previous biochemical data indicating that cGK in mammalian brain is concentrated in cerebellum (10–13, 18), and they provide direct evidence that within cerebellum cGK is concentrated in a specific population of neurons: the Purkinje cells. A specific association of cGK with Purkinje cells had previously been suggested by the observation that, in mutant mice specifically lacking Purkinje cells, cerebellar cGK levels were greatly reduced (13, 18). No other immunoreactive nerve cells have been identified either in cerebellum or in any other brain area examined. Interestingly, the only nerve-cell-specific substrate so far identified for cGK (14–17) is greatly enriched in the Purkinje cells of the cerebellum (ref. 13 and unpublished observations). In addition, the high levels of cGMP found in cerebellum compared to other brain regions appear to be due primarily to the Purkinje cells (7–9).

Although our results do not exclude the possibility that low levels of cGK may be present in other types of neurons, they do indicate that Purkinje cells have unique biochemical characteristics. An important role of the cGMP system in the physiology of Purkinje cells has been suggested by pharmacological experiments. For instance, activation or inhibition of Purkinje cells, induced by drugs and putative neurotransmitters, is paralleled by an increase or a decrease, respectively, of cerebellar cGMP levels (4, 28, 29).

Immunoreactivity was found on smooth muscle cells of the cerebral vasculature. This finding is consistent with our RIA and immunohistochemical results, which indicate that smooth muscle cells of several tissues examined are rich in cGK (23). Further investigation is required to establish the extent to which smooth muscle cells of the vessels contribute to the cGK content of the brain. Because only low levels of cGK are detectable biochemically in regions other than cerebellum, the possibility must be considered that much of this cGK might be due to vessels.

The morphological distribution of cGK immunoreactivity both in Purkinje cells and in smooth muscle cells is consistent with a predominant localization of cGK in the cytosol. This is especially evident in Purkinje cells, where immunostaining can be observed in all regions of the cell in spite of the presence of different cellular domains with distinct functions (dendrites, perikarya, axons, and nerve terminals). It is interesting in this respect that the one known endogenous neuronal substrate for cerebellar cGK is a cytosolic protein (15).

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