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Immunohistochemical localization of guanylate cyclase within neurons of rat brain

(cyclic GMP/caudate–putamen/neocortex/immunofluorescence/cerebellum)

MARJORIE A. ARIANO*, JOHN A. LEWICKI†, HARVEY J. BRANDWEIN†, AND FERID MURAD†‡

*Department of Anatomy and Neurobiology, University of Vermont College of Medicine, Burlington, Vermont 05405; and †Departments of Medicine and Pharmacology, Stanford University, Palo Alto Veterans Administration Medical Center, Palo Alto, California 94304

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ABSTRACT **The immunohistochemical localization of guanylate cyclase [GTP pyrophosphate-lyase (cyclizing), EC 4.6.1.2] has been examined in rat neocortex, caudate–putamen, and cerebellum by using specific monoclonal antibodies. Immunofluorescence could be seen within somata and proximal dendrites of neurons in these regions. A nuclear immunofluorescence reaction to guanylate cyclase was characteristically absent. The staining pattern for guanylate cyclase was coincident with previously described localizations of cyclic GMP immunofluorescence within medium spiny neurons of the caudate–putamen and pyramidal cells of the neocortex. Cerebellar guanylate cyclase immunoreactivity was primarily confined to Purkinje cells and their primary dendrites, similar to the pattern reported for cyclic GMP-dependent protein kinase localization. Guanylate cyclase immunofluorescence was abolished when the monoclonal antibodies were exposed to purified enzyme prior to incubation of the tissue slices or when control antibody was substituted for the primary antibody. Immunohistochemical localization of cyclic AMP in these same tissues was readily distinguished from that of guanylate cyclase or cyclic GMP, showing uniform fluorescence throughout the cell bodies of neurons and glial elements.**

Guanylate cyclase [GTP pyrophosphate lyase (cyclizing), EC 4.6.1.2] catalyzes the formation of guanosine 3′,5′-monophosphate (cyclic GMP) from GTP. Two forms of the enzyme, one soluble and the other membrane-associated, have been described in most tissues studied (1). Characterization of the enzyme in murine brain (2) demonstrates comparable enzyme activity in the neocortex and cerebellum and highest total guanylate cyclase activity in the caudate–putamen complex. Subcellular fractionation of mouse (2) and rat (3) caudate–putamen homogenates shows the specific activity of the soluble form of the enzyme to be greater than that of the particulate fraction. It has been hypothesized that cyclic GMP has an important role in the mediation of cholinergic-muscarinic receptor actions (4–7), glutamate neurotransmission (8), and the generation of seizure activity (9) in the nervous system.

Another experimental approach, immunohistochemical localization of cyclic GMP in various brain regions, has demonstrated accumulation of this cyclic nucleotide in neuroglia (10, 11), in stellate and basket neurons of the cerebellum (10), in postganglionic neurons of the superior cervical ganglion (12, 13), and predominantly within neurons of the caudate–putamen and the neocortex (14). Immunocytochemistry in the latter regions demonstrated cyclic GMP accumulation at the subsynaptic terminal density and adjacent cytoplasm in type 1 synapses (14). Cyclic GMP has been proposed to exert its physiological actions through phosphorylation of a cyclic GMP-dependent protein kinase (15). Cyclic GMP-dependent protein kinase activity is extremely elevated in the cerebellum (16), and immunohistochemical localization of this enzyme has been demonstrated within Purkinje cells and their processes (17). Cyclic GMP phosphodiesterase (EC 3.1.4.17), the enzyme that hydrolyzes cyclic GMP to 5′-GMP, has been detected cytochemically at the subsynaptic density of axospinous synapses of the rat caudate–putamen complex and neocortex (18). A preferential localization of soluble guanylate cyclase in neurons has been described in disrupted rat brain (19) and after kainic acid production of lesions of the rat caudate–putamen (3).

The present work describes the immunohistochemical localization of guanylate cyclase in three central nervous system areas by using four different monoclonal antibodies directed against the soluble form of rat lung enzyme. The antibodies crossreact avidly with soluble guanylate cyclase from all rat tissues examined, including brain, and may partially interact with the particulate enzyme form (20). Specific localization of guanylate cyclase was determined by using the fluorescein double-antibody technique (21). The enzyme was visualized within the cytoplasm and proximal dendrites of neurons of the rat caudate–putamen, neocortex, and cerebellum. In addition, the immunohistochemical localization of cyclic GMP and cyclic AMP was concurrently visualized in these same regions.

METHODS

Male Sprague–Dawley rats (200–250 g) provided experimental tissue. Animals were decapitated, the brain was removed *en toto*, and a coronal block was made caudal to the hypophyseal stalk. The cut surface was frozen to a brass mounting chuck and stored at −25°C in a cryostat. Coronal 6- to 8-μm sections of the rostral caudate–putamen complex and overlying neocortex at the level of +3.4 mm, according to the brain atlas of Pellegrino *et al.* (22), were taken for immunohistochemical analysis. Tissue sections were thaw-mounted onto chrome alum-coated slides, briefly air dried, and washed for 15–30 min at room temperature in 0.05 M sodium phosphate-buffered, 0.9% saline, pH 7.2 (P_i/NaCl). Four monoclonal antibodies directed against soluble guanylate cyclase were applied to various tissue sections at concentrations of 0.01–0.02 μg/ml in P_i/NaCl and incubated 8–22 hr at 4°C in a moisture chamber. The tissue sections were washed 1 hr in P_i/NaCl after the primary antibody incubation. The monoclonal antibodies bound to guanylate cyclase were visualized by application of an appropriate secondary fluorescein isothiocyanate conjugate of goat anti-mouse IgG (γ-chain specific) or IgM (μ-chain specific) (Cappel Laboratories, Cochranville, PA) for 1 hr at 4°C at a 1:100 dilution. Unbound secondary antibody was washed off the slides with P_i/NaCl. Slides were dried and viewed under glycerol immersion at ×25.

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Abbreviation: P_i/NaCl, phosphate-buffered saline.

‡ To whom reprint requests should be addressed at: Dept. of Medicine, Veterans Admin. Hospital, 3801 Miranda Ave., Palo Alto, CA 94304.

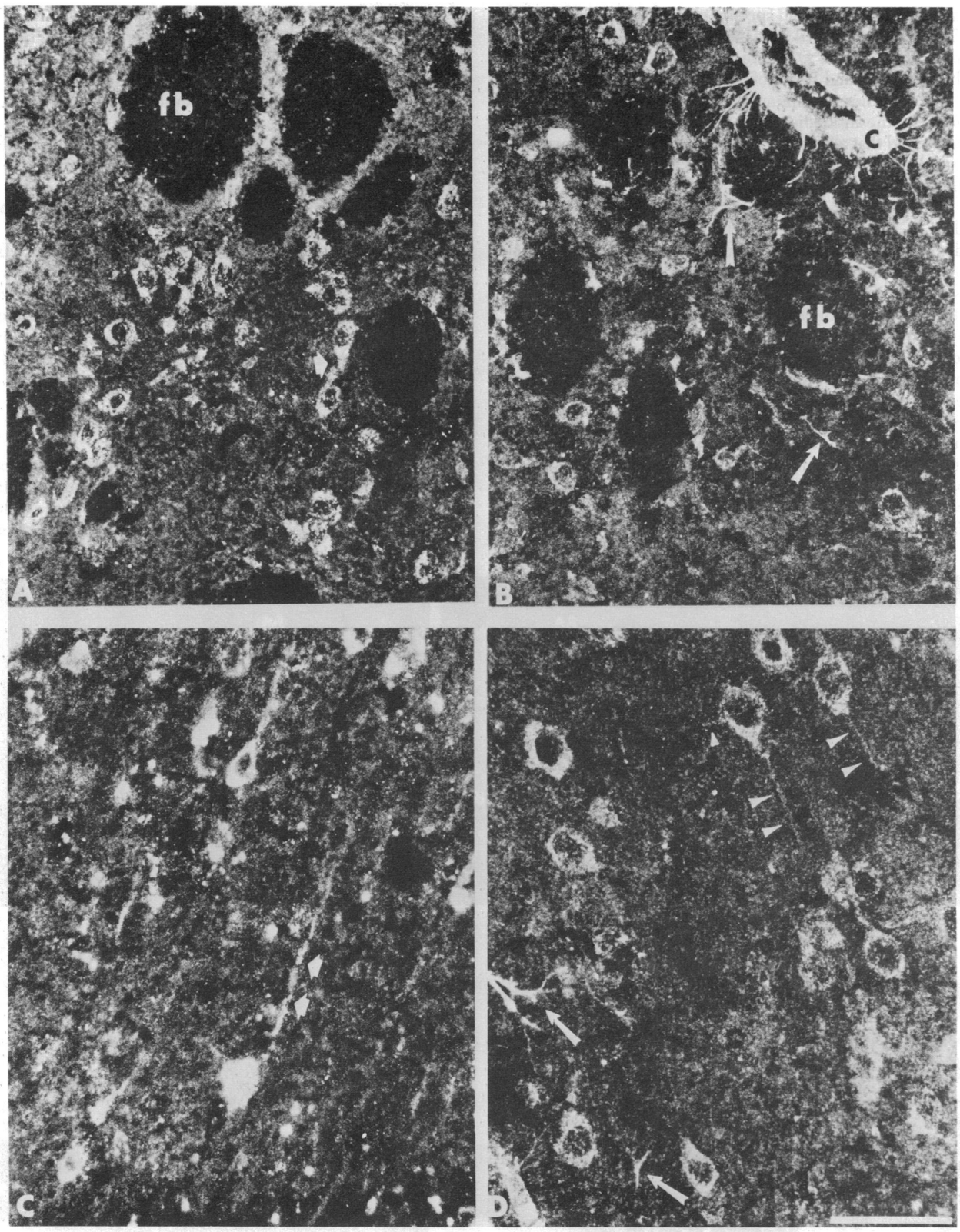

FIG. 1. Immunohistochemistry in rat brain. (*A*) Guanylate cyclase immunofluorescence in caudate–putamen. Cytoplasmic immunoreactivity is readily visualized in cells 15–20 μm in diameter. Proximal dendrites are reactive for guanylate cyclase (wide arrow). Lack of fluorescence in neuronal nuclei and fiber bundles (fb) is characteristic. Monoclonal antibody B_4 was used. (*B*) Cyclic GMP immunofluorescence in caudate–putamen. Cytoplasmic staining is apparent in 15- to 20-μm-diameter medium spiny neurons. Fibrous astrocytic elements are reactive for cyclic GMP (arrows). The c denotes a capillary with reactive astrocytic endfeet; fb, a nonreactive fiber bundle. (*C*) Guanylate cyclase immunofluorescence in neocortex. Cytoplasmic immunoreactivity is readily visualized in large neurons. Proximal dendrites are reactive for guanylate cyclase (wide arrows). Lack of nuclear fluorescence is characteristic. Monoclonal antibody D_1 was used. (*D*) Cyclic GMP immunofluorescence in neocortex. Cytoplasmic staining of pyramidal cells and apical and basilar dendrites (arrowheads) is readily seen. Fibrous astrocytic elements are reactive for cyclic GMP (arrows). Calibration bar, 50 μm.

Antibody production and specificity has been described (20, 23). The four monoclonal antibodies used in the study, designated B_1, B_2, B_4, and D_1, have been characterized in detail. Antibodies B_1, B_2 and B_4 are of the IgG1 subclass, and D_1 is an IgM. Prior studies examining the interaction of crude tissue extracts with the antibodies immobilized on solid supports have demonstrated that all four antibodies specifically recognize soluble guanylate cyclase from a variety of tissues (24).

The specificity of staining for guanylate cyclase was tested with the following controls: (*i*) use of a control monoclonal antibody (i.e., no anti-guanylate cyclase activity) as a primary antibody; this IgG1 antibody was used at the same concentration as the specific monoclonal antibodies; (*ii*) use of nonimmunized mouse immunoglobulin for the primary antibody; (*iii*) prior incubation of anti-guanylate cyclase antibodies with purified guanylate cyclase (30 min at 37°C, followed by 12 hr at 4°C) and subsequent immunohistochemical processing using a 1:100 dilution; and (*iv*) increasing dilutions of the anti-guanylate cyclase antibodies from 0.1 μg/ml through 0.0001 μg/ml in P_i/NaCl. The staining reaction was considered specific when no immunofluorescence resulted after any of these four treatments. The immunohistochemical methodological control was application of goat anti-mouse IgG or goat anti-mouse IgM (Cappel Laboratories, Cochranville, PA) at 1:10 through 1:1000 dilutions in P_i/NaCl for 8–22 hr at 4°C.

Cyclic GMP and cyclic AMP antisera were raised in rabbits according to the method described by Spruill and Steiner (21) and have been extensively characterized (13, 14). These antisera were used at a dilution of 1:500 in P_i/NaCl for 10–22 hr at 4°C or 1:50 for 2 hr at 4°C. Secondary antibody was fluorescein isothiocyanate-conjugated goat anti-rabbit IgG diluted 1:50, and applied for 30–60 min at 4°C. After each antibody incubation, tissues were rinsed in P_i/NaCl for 1 hr at room temperature and then examined at ×25 under glycerol immersion.

Tissues were examined with a Zeiss Photomicroscope 3 equipped with an HBO 100 ultraviolet light source. The primary filter (excitation) was 420 nm and the secondary filter (barrier) was 485 nm. Photomicrography of tissues was recorded on Tri-X (Kodak) or Ilford HP5 (CIBA–Geigy) black and white film pushed to ASA 800 and developed with D-19 developer (Kodak) to increase the contrast of the photographic negatives. Exposures have been made by using the automatic mode of the Photomicroscope 3 camera.

RESULTS

Monoclonal antibodies to soluble guanylate cyclase have been used to determine the cellular location of this enzyme within the rat neocortex, caudate-putamen complex, and cerebellum. Immunofluorescence was readily visualized as an even staining

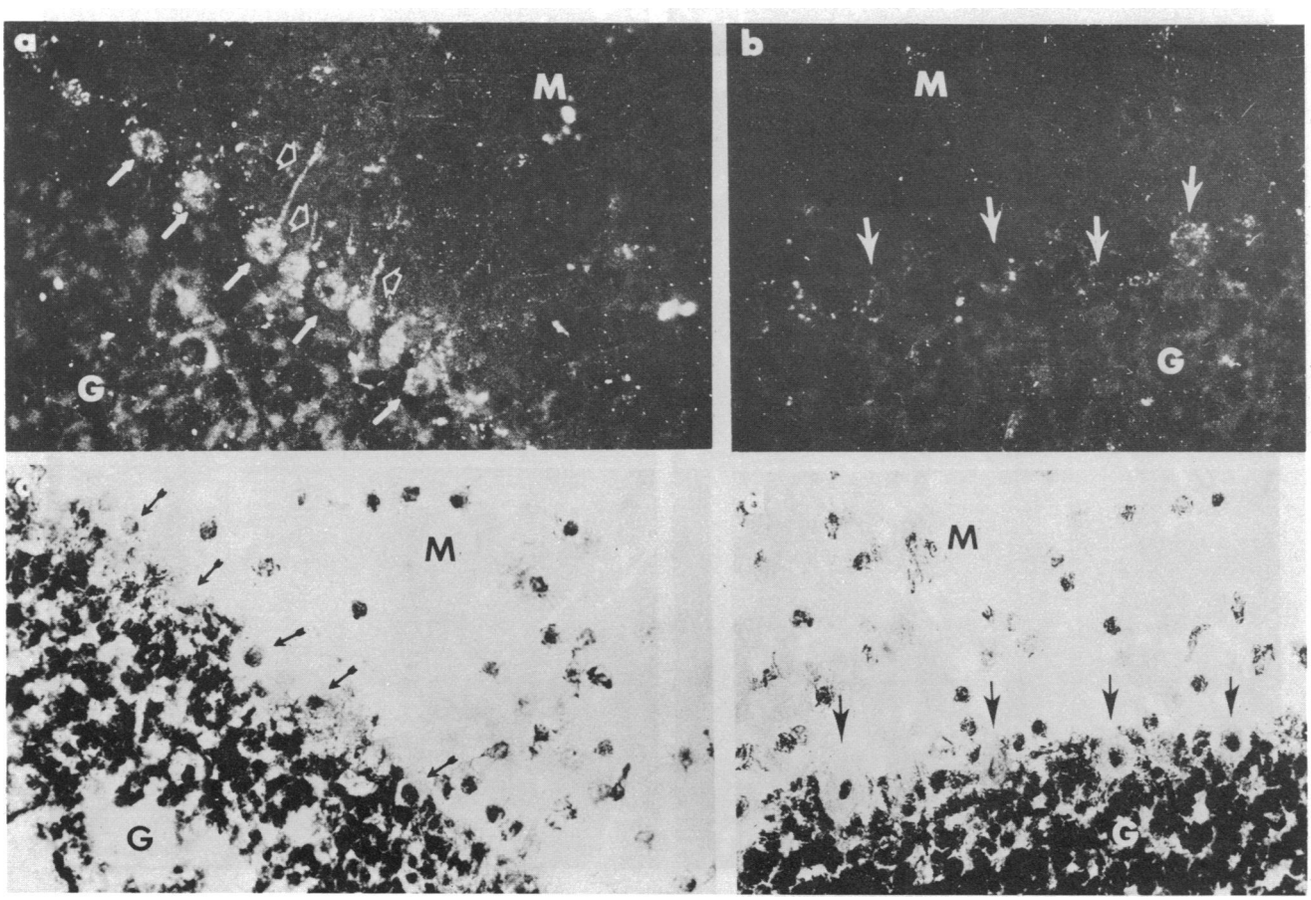

FIG. 2. Immunohistochemistry in cerebellum. (*a*) Guanylate cyclase immunohistofluorescence in Purkinje somata (filled arrows) and primary dendrites (open wide arrows). Some immunoreactive cells are apparent in the granular layer (G). Stained elements are not present in the molecular layer (M). (*b*) Guanylate cyclase staining in cerebellum after preabsorption block of the antisera prior to immunohistochemical processing. Arrows denote four Purkinje cell somata, and the molecular layer (M) and the granular layer (G) are labeled. Monoclonal antibody B_2 was used in *a* and *b*. (*c*) Toluidine blue counterstain of cerebellar tissue demonstrating all the cell bodies within the field of *a*. Arrows point to the same five Purkinje cell bodies in *a*. Molecular (M) and granular (G) layer neurons and glia are readily seen. (*d*) Toluidine blue counterstain of cerebellum section seen in *b*. Arrows denote the same four Purkinje cell bodies as seen in *b*. Neurons and glia in the molecular (M) and granular (G) layer are readily seen. (×620.)

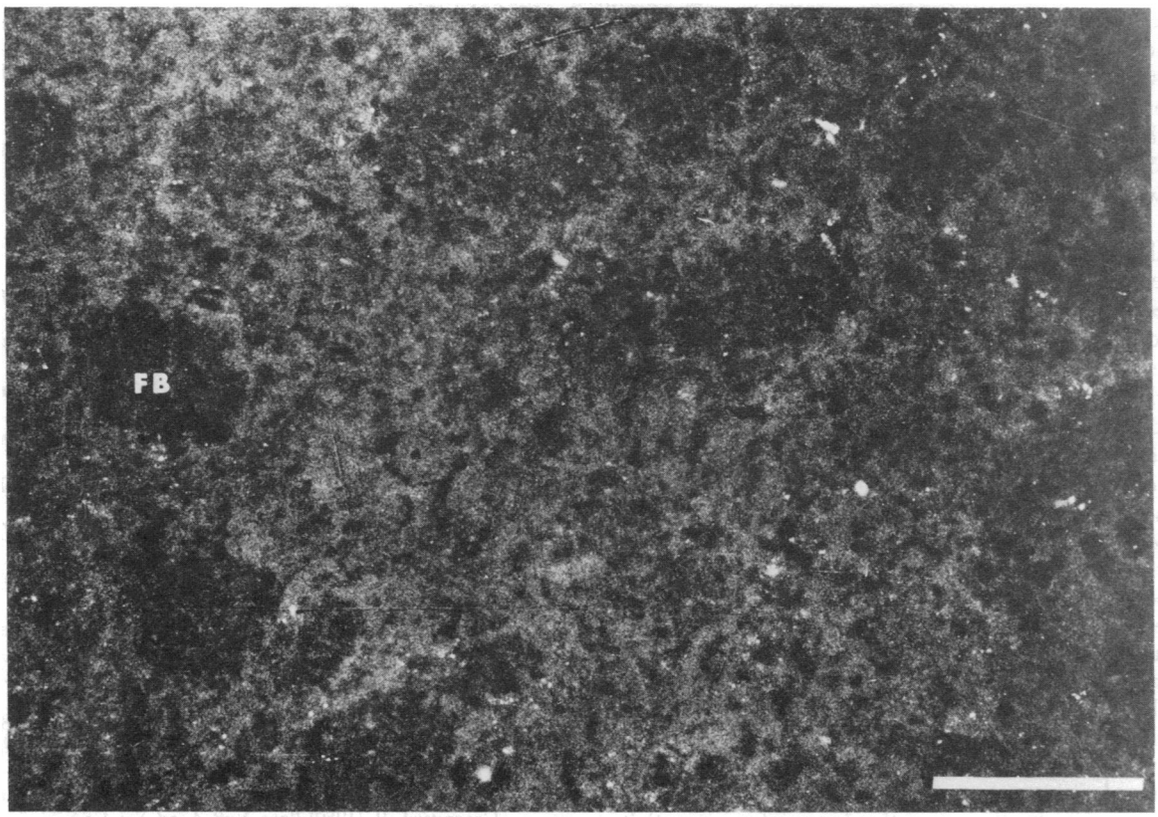

FIG. 3. Immunohistochemical staining of rat caudate–putamen. The primary antibody was a control monoclonal antibody that did not exhibit anti-guanylate cyclase activity as assessed with immunoprecipitation. Sparse deposits of autofluorescent lipofuscin granules are visible. FB, fiber bundle. Calibration bar, 50 μm.

within somata and proximal dendrites of neurons in these brain regions. Elements demonstrating guanylate cyclase immunoreactivity in the caudate–putamen (Fig. 1*A*) correspond in size and dimensions to the medium spiny neuron (25). The identification of these cells was confirmed by using toluidine blue counterstain. The larger pyramidal neurons of layers 3 and 5 of the neocortex and their apical dendrites (wide arrows, Fig. 1*C*) were reactive in guanylate cyclase immunohistochemistry. The identification of the positively fluorescent elements was based on toluidine blue counterstaining and the distinct cytoarchitecture of this neuronal type (26). Purkinje cells and their primary dendritic tree were readily discerned as containing guanylate cyclase immunoreactivity (Fig. 2*a*). Immunofluorescence staining was also apparent within stellate and basket neurons of the granular layer. Toluidine blue counterstain (Fig. 2*c*) demonstrates all of the cells present in the field and illustrates the identity of the various cell types. Nuclear staining was characteristically absent in the reactive cells in all experimental tissue examined. Qualitatively similar staining patterns were observed after immunohistochemistry with all four different monoclonal antibodies. The fluorescence pattern seen in guanylate cyclase immunohistochemical assessment was coincident with cyclic GMP localization in these central nervous system areas (Fig. 1 *B* and *D*). In addition to its neuronal localization, cyclic GMP immunoreactivity could also be seen within processes of fibrous astrocytes (arrows in Fig. 1 *B* and *D*), and in Bergmann glia of the cerebellum (data not shown; ref. 11), but this cellular element was not reactive for guanylate cyclase. There are several explanations for this disparity. Astrocytes may absorb cyclic GMP from the extracellular milieu, and therefore the nucleotide may not be synthesized in these cells. Alternatively, astrocytes may contain predominately particulate guanylate cyclase that is not being detected by the antibodies.

Immunofluorescence was abolished when the monoclonal antibodies were first exposed to purified guanylate cyclase as described in *Methods* (Fig. 2*b*). The molar enzyme-to-antibody ratio was 5. Specific guanylate cyclase staining was also absent when control monoclonal antibody (i.e., no anti-guanylate cyclase immunoreactivity) was used as the primary antibody (Fig. 3). Immunohistochemical staining for cyclic AMP was quite different from either cyclic GMP or guanylate cyclase. Fluorescent staining for this cyclic nucleotide was uniformly observed in both neuronal and glial elements (data not shown).

DISCUSSION

Immunohistochemical methods have been used to determine the cellular localization of guanylate cyclase in intact brain tissue. Four monoclonal antibodies, all of which have been well characterized (refs. 20, 23, and 24 and *Methods*), were used to visualize the distribution of the enzyme. Although the antibodies were produced against the soluble rat lung enzyme, all have been demonstrated previously to crossreact with the soluble brain enzyme by using immunoprecipitation (20). In addition, competitive binding experiments have demonstrated recently that the four monoclonal antibodies are distinct and recognize several different specific determinants on the enzyme (ref. 24 and unpublished observations). Thus, the observation that all three IgG and the IgM monoclonal antibodies give similar qualitative immunofluorescent staining patterns is strong evidence that they are specifically staining guanylate cyclase. Furthermore, the numerous controls reported here substantiate the specificity of the immunohistochemical method.

The localization of guanylate cyclase was within neuronal perikarya and proximal dendrites of the rat caudate–putamen

complex, neocortex, and cerebellum. Definitive identification of these cell types in this heterogeneous tissue has been accomplished through the use of toluidine blue counterstain and the characteristic cytoarchitecture of the neurons previously described by using the Golgi technique for these three brain regions (25–27). The cellular immunofluorescence of guanylate cyclase was virtually identical to that of its product cyclic GMP, but was readily distinguished from cyclic AMP immunohistochemical staining. The coincident localization of the cyclic GMP-dependent protein kinase in cerebellar Purkinje neurons described by Lohmann and coworkers (17) adds evidence for the selectivity of the cyclic GMP system in this area of brain. The recent report of Zwiller *et al.* (28) of ubiquitous localization of guanylate cyclase by polyclonal antibody immunohistochemistry in cerebellum is in contrast to reports of other investigators, which support a cellular selectivity of cyclic GMP (10, 11, 13, 14), cyclic GMP-dependent protein kinase (17), and cyclic GMP phosphodiesterase (2, 18), as well as being in conflict with the present description of guanylate cyclase localization. There is no obvious way to reconcile this disparity at present other than to ascribe it to methodological differences and our use of more selective monoclonal antibodies to guanylate cyclase.

Immunocytochemical localization of guanylate cyclase will determine the coincidence of the enzyme and cyclic GMP at subcellular sites. It is not clear at present if we are staining the soluble or the particulate form of guanylate cyclase, or both. Although the antibodies were prepared against the soluble enzyme, they also appear to partially crossreact with the particulate enzyme (20). Furthermore, the degree of crossreactivity is increased when the particulate enzyme is immobilized (e.g., on a solid support or membrane). Therefore, it is possible that we are measuring particulate enzyme as well as the soluble enzyme that remains after tissue preparation. Immunocytochemical data, along with specific antisera to the particulate enzyme form, are required to resolve this issue. At present, it can be concluded only that we are visualizing a pool of enzyme that correlates in part with intracellular pools of cyclic GMP. Further, the predominantly neuronal location of cyclic GMP, cyclic GMP phosphodiesterase, cyclic GMP-dependent protein kinase, and guanylate cyclase suggest a role for cyclic GMP in neurotransmission.

Note Added in Proof. In our subsequent immunohistochemical studies with monoclonal antibodies B_4 and D_1 in rat superior cervical ganglion, fluorescent staining was confined to only the cytoplasm and processes of postganglionic neurons.

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1. Kimura, H. & Murad, F. (1975) *Metabolism* **24**, 439–445.
2. Greenberg, L. H., Troyer, E. A., Ferrendelli, J. A. & Weiss, B. (1978) *Neuropharmacology* **17**, 737–745.
3. Walaas, I. (1981) *J. Neurochem.* **36**, 233–241.
4. Weight, F. F., Petzold, G. L. & Greengard, P. (1974) *Science* **186**, 942–944.
5. Lee, T. P., Kuo, J. F. & Greengard, P. (1972) *Proc. Natl. Acad. Sci. USA* **69**, 3287–3291.
6. Stone, T. W. & Taylor, D. A. (1977) *J. Physiol. (London)* **266**, 523–543.
7. Hanley, M. R. & Iversen, L. L. (1978) *Mol. Pharmacol.* **14**, 246–255.
8. Mao, C. C., Guidotti, A. & Costa, E. (1974) *Brain Res.* **79**, 510–514.
9. Ferrendelli, J. A., Blank, A. C. & Gross, R. A. (1980) *Brain Res.* **200**, 93–103.
10. Chan-Palay, V. & Palay, S. L. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 1485–1488.
11. Cumming, R., Arbuthnott, G. & Steiner, A. L. (1979) *J. Cyclic Nucleotide Res.* **5**, 463–467.
12. Kebabian, J. W., Bloom, F. E., Steiner, A. L. & Greengard, P. (1975) *Science* **190**, 157–159.
13. Briggs, C. A., Ariano, M. A. & McAfee, D. A. (1981) *Soc. Neurosci.* **7**, 919.
14. Ariano, M. A. & Matus, A. I. (1981) *J. Cell Biol.* **91**, 287–292.
15. Greengard, P. (1980) *Cyclic Nucleotides, Phosphorylated Proteins, and Neuronal Function* (Raven, New York).
16. Schlichter, D. J., Detre, J. A., Aswad, D. W., Chehrazi, B. & Greengard, P. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 5537–5541.
17. Lohmann, S. M., Walter, U., Miller, P. E., Greengard, P. & DeCamilli, P. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 653–657.
18. Ariano, M. A. & Appleman, M. M. (1979) *Brain Res.* **177**, 301–309.
19. Goridis, C. & Morgan, I. G. (1973) *FEBS Lett.* **34**, 71–73.
20. Brandwein, H. J., Lewicki, J. A. & Murad, F. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 4241–4245.
21. Spruill, W. A. & Steiner, A. L. (1979) *Adv. Cyclic Nucleotide Res.* **10**, 169–186.
22. Pellegrino, L. J., Pellegrino, A. S. & Cushman, A. J. (1979) *A Stereotaxic Atlas of the Rat Brain* (Plenum, New York).
23. Lewicki, J. A., Brandwein, H., Waldman, S. A. & Murad, F. (1980) *J. Cyclic Nucleotide Res.* **6**, 283–296.
24. Lewicki, J. A., Brandwein, H., Waldman, S. A. & Murad, F. (1981) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **40**, 664 (abstr.).
25. DeFiglia, M., Pasik, P. & Pasik, T. (1976) *Brain Res.* **114**, 245–256.
26. Jones, E. G. & Powell, T. P. S. (1970) *Philos. Trans. R. Soc. London Ser. B* **257**, 1–11.
27. Palay, S. L. & Chan-Palay, V. (1973) *Cerebellar Cortex: Cytology and Organization* (Springer, Berlin).
28. Zwiller, J., Ghandour, M. S., Revel, M. O. & Basset, P. (1981) *Neurosci. Lett.* **23**, 31–37.