ABSTRACT  Guanosine 3’,5’-cyclic monophosphate (cGMP) immunoreactivity in the rat’s cerebellum was studied with light and electron microscopy by the indirect fluorescence method and the peroxidase-antiperoxidase method. Labeled cells included neuroglial cells in the cerebellar cortex, white matter, and deep nuclei; some stellate and basket cells in the cortex; and some large neurons in the deep nuclei. No evidence was found for sagittal microzonation in the cGMP distribution. In the labeled cells, cGMP immunoreactive sites were localized to surface membranes, organelles, and the cytoplasmic matrix. Specificity was indicated by the same pattern of labeling after treatment with cGMP immunoglobulin that had been adsorbed with adenosine 3’,5’-cyclic monophosphate (cAMP) and by the failure to label after treatment with normal rabbit serum or with cGMP immunoglobulin that had been adsorbed with 1 mM cGMP. Cerebella treated with cAMP antiserum, however, showed immunoreactivity in Purkinje cells, granule cells, and Golgi cells in addition to neuroglia in cortex and deep nuclei. Sequential norepinephrine and glutamate superfusions generally intensified cGMP immunoreactivity, not only in neuroglial cells but also in the background. Under these conditions some Purkinje cells and some granule cells were also labeled. Increased cGMP immunoreactivity was also obtained by treatment with harmaline, γ-aminobutyric acid and aminooxyacetic acid, muscimol, γ-aminobutyric acid, or apomorphine in order of decreasing effectiveness. Serotonin and colchicine produced no detectable increase of cGMP immunoreactivity above normal, and diazepam and sodium pentobarbital decreased it. In these experiments, diethyl ether was preferable to sodium pentobarbital for anesthesia on account of the depressive action of the latter on cGMP immunoreactivity. Thus, drugs that increase cerebellar activity enhance cGMP levels, whereas those that decrease cerebellar activity decrease cGMP levels. However, it is not clear whether these fluctuations in cGMP levels are a direct consequence of neurotransmitter function or are sequelae to other related events. The present study suggests that some neurons and many neuroglial cells are the major sites of cGMP in the cerebellum.

The mammalian cerebellum contains unusually high concentrations of endogenous guanosine 3’,5’-cyclic monophosphate (cGMP) (1). Experiments on brain slices, cultures, and in vitro preparations suggest that cGMP may be associated with a variety of possible neurotransmitter substances in mechanisms that are not understood. Selective changes of cerebellar cGMP independent of adenosine 3’,5’-cyclic monophosphate (cAMP) can be produced by a number of agents and conditions such as alcohol (2), excitatory and inhibitory transmitter substances (3, 4), decapitation (5), and benzodiazepines (6). Considerable speculation marks the interpretation of the correlations among cGMP levels, putative transmitter substances, and their possible localization in cerebellar neurons, particularly Purkinje cells (refs. 7 and 8; reviewed in ref. 9, p. 229). The presence of cGMP in Purkinje cells has not been demonstrated (10). This study presents cytopharmacological evidence from immunocytochemistry for the cellular and subcellular location of cGMP in some cerebellar neurons and particularly in neuroglial cells.

MATERIALS AND METHODS

Adult 200–300 g Sprague–Dawley rats, the indirect immunofluorescence method (11), and the peroxidase-antiperoxidase (PAP) method (12) for light and electron microscopy were used. Rabbits and guinea pigs were raised against the 2’-O-succinyl derivative of cGMP or cAMP conjugated to limpet hemocyanin (13). More than 50% of 2’-O-succinyl-cGMP ([125]iodotyrosine methyl ester derivative) or 2’-O-succinyl-cAMP ([125]iodotyrosine methyl ester derivative) (<0.01 pmol) was bound at a serum dilution of 1:2000. Specificity was determined by radioimmunoassay. The antisera raised against cGMP gave 20–30% displacement of bound [125]labeled cGMP with 5 fmol of acetylated cGMP. Acetylated cAMP did not produce significant displacement up to 1000 fmol. Corresponding experiments with antisera raised against cAMP produced the equivalent appropriate result, indicating its specificity. The immunoglobulin (IgG) fractions of these antisera were used. The basic cytochemical methods have been described (14). Cryostat sections (10 μm thick) of cerebella from formaldehyde-perfused brains and unfixed cerebella frozen in liquid nitrogen 1–2 min after decapitation were treated with the IgG and stained by the immunofluorescence method, using a fluorescein isothiocyanate-conjugated goat IgG. Vibratome sections (20–30 μm thick, cut in cold 0.05 M Tris-HCl buffer, pH 7.6) of cerebella from perfused brains were treated sequentially with cGMP IgG (18 hr at 4°C in a humid environment, dilution 1:100 in 0.5% Triton X-100/0.05 M Tris-HCl buffer, pH 7.6), goat-anti-rabbit IgG, PAP antiserum, and diaminobenzidine with hydrogen peroxide, prior to postfixation in 2% osmium tetroxide, dehydration with methanol, and embedding in epoxy resin. These sections were then examined with the light microscope and subsequently thin-sectioned in serial order for electron microscopy. No counterstains were used for electron microscopy. Two forms of anesthesia were employed and their results were compared: 2% sodium pentobarbital (0.1 ml/100 g of body weight) injected intraperitoneally and diethyl ether by inhalation. Two fixatives were used in the perfusions, either 4% (wt/vol) formaldehyde in Na phosphate buffer, pH 7.4, or 4% formaldehyde and 0.25% glutaraldehyde in phosphate buffer, pH 7.4, both at 4°C; each was preceded by a wash with cold Ca²⁺-free Tyrode’s buffer.

Nine separate sets of experiments were performed, on groups of three rats each, using drugs to alter cGMP levels detectable by immunocytochemistry. All animals were perfused with fixative at the stated intervals after drug administration (1).
Diazepam, 25 mg/kg of body weight, intraperitoneal, 30 min prior to perfusion; (ii) muscimol, 10 mg/kg of body weight, intravenous, 30 min prior to perfusion; (iii) apomorphine, 5 mg/kg of body weight, intraperitoneal, 5 min prior to perfusion; (iv) harmaline, 40 mg/kg of body weight, intraperitoneal, 30 min prior to perfusion; (v) γ-aminobutyric acid (GABA) 10 µM, 2-µl intracerebellar injections, 5–7 min prior to perfusion; (vi) GABA, 10 µM, 2-µl intracerebellar injections, 5–7 min prior to perfusion with aminoxyacetic acid, 10 mg/250 g of body weight, intraperitoneal, 30 min prior to perfusion; (vii) norepinephrine, 300 µM, and sodium L-glutamate, 3 mM in sterile saline at 37°C, superfused in sequence for 10 min each by using a push–pull cannula and agar superfusion chamber over the cerebellar cortical surface; (viii) serotonin, 0.1 µM (250 µl), intraventricular infusion over 3 hr with monoamine oxidase inhibition (clorgyline, 10 mg/100 g of body weight); (ix) colchicine (3 µg/µl), intracerebellar injection, 2 µl per electrode track, and 25 µl intraventricularly 24 hr prior to perfusion.

Parasagittal and transverse sections from three cerebellar regions (vermis, hemisphere, and paravermis including deep nuclei) in each animal were processed. Tissues from the separate experiments and controls were handled at the same time in order to reduce technical differences. Controls for specificity included: cAMP antisera; cyclic GMP IgG adsorbed overnight with 1 mM cGMP; cGMP IgG adsorbed overnight with 1 mM cAMP; and normal rabbit serum. The resulting material was studied with the light microscope; neurons and neuroglia with cGMP immunoreactivity were identified in the cerebellar cortex, white matter, and deep nuclei. The distribution and intensity of immunoreactivity were determined for specimens in all experiments using a scale of 1+ to 4+ for increasing amounts and intensities of reactions. Electron microscopy was conducted on serial sections of six specimens obtained from normal, untreated, anesthetized animals. The sections were treated with cGMP IgG, and cGMP IgG adsorbed with cGMP was used for controls.

**RESULTS**

cGMP immunoreactivity was greater: (i) in the cerebella from animals anesthetized with ether compared to sodium pentobarbital; (ii) in the more sensitive PAP method compared to immunofluorescence; (iii) in tissues fixed in formaldehyde without glutaraldehyde compared to unfixed frozen material. The presence of glutaraldehyde in the primary fixative enhanced morphological preservation for electron microscopy but reduced immunoreactivity. Colchicine treatment did not enhance cGMP immunoreactivity. In the molecular layer, some stellate and basket somata (approximately 30%) were cGMP immunoreactive. The Golgi epithelial neuroglial somata surrounding Purkinje cells and their radial fibers (15) displayed the most intense immunoreactivity. Purkinje cell somata remained unreactive with all modes of fixation and drug manipulations attempted except for the superfusion with norepinephrine and glutamate. Neuroglial cells in the granular layer and their processes (15, 16) were immunoreactive, whereas Golgi neurons and granule cell somata were not. In the white matter, some oligodendroglial cells and some axons were immunoreactive. In the cerebellar nuclei, neuroglial cells were generally reactive, whereas the neurons were not and the total amount of cGMP immunoreactivity was considerably lower.

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**Fig. 1.** Light micrographs of immunoreactive neuroglial and neuronal elements of the cerebellar cortex visualized by cGMP IgG (a–d, f–i) and cAMP antisera (j) by using the PAP method. (a and b) Tissue from normal, untreated, ether-anesthetized animal showing cGMP immunoreactivity in glial cells surrounding Purkinje cells (PC) and in the granular layer (arrows). (a, x120; b, x480.) (c and d) Ether-anesthetized animal treated with apomorphine. (c, x120; d, x480.) (e) Control tissue reacted with cGMP IgG preadsorbed with 1 mM cGMP. (x480.) (f and g) Normal untreated animal anesthetized with sodium pentobarbital. (f, x120; g, x480.) (h and i) cGMP immunoreactivity in tissue from animal anesthetized with ether and treated with harmaline. (h, x120; i, x480.) (j) Normal, untreated, ether-anesthetized animal treated with cAMP antisera. (X120.)
in the cerebellar nuclei than in the cortex. Intracerebellar injections of GABA with or without aminooxyacetic acid induced cGMP immunoreactivity in some large neurons. Labeled cells and their processes were randomly scattered in the cerebellum and no indication of sagittal microzonation was observed in cGMP distribution (17).

Selective changes in the location and intensity of cGMP immunoreactivity were detectable at a cellular level on ad-
munication of various drugs. Superfusion with norepinephrine and glutamate caused the greatest increase (18). Under these conditions superficial cortical folia directly perfused showed, in addition to the reactive neuronal and glia elements usually found, reactive Purkinje cell somata (up to 80% of the population) and dendrites, reactive granule cell somata (up to 50%), and Golgi neurons (30%). Because these reactions all occurred in an unusually high background, it is difficult to assess their significance. In the cerebella of animals treated with harmaline, GABA with aminoxyacetic acid, muscimol, GABA, or apomorphine, cyclic GMP immunoreactivity in neuroglial cells generally was increased above that in normal animals, but it was lower in the neuroglial cells of the Purkinje cell layer. Stellate and basket neurons were more reactive after GABA and harmaline treatment than in untreated animals. Intraventricular infusions of serotonin produced cGMP immunoreactivity equal in intensity and distribution to that of the normal untreated animal, and diazepam and sodium pentobarbital treatment reduced cGMP immunoreactivity below normal levels (see Fig. 1 a-d and f-i). No specific staining was obtained when normal rabbit serum and cGMP IgG adsorbed with cGMP were used. cGMP IgG adsorbed with cAMP showed a labeling pattern no different from that obtained with unadsorbed cGMP IgG. Comparative studies with cAMP antibody repeatedly indicated immunoreactivity in Purkinje cells (Fig. 1f), granule cells (19), stellate and basket cells, Golgi cells, numerous Golgi epithelial neuroglial cells and their processes, and some large and small neurons in the deep cerebellar nuclei. Because these results differ significantly from the sites of cGMP immunoreactivity, the data indicate that the cytochemical activities of cGMP and cAMP antisera are distinguishable.

Electron microscopy confirmed the findings of light microscopy that the majority of labeled cells and processes were neuroglial: Golgi epithelial cells in between Purkinje cells, their processes the Bergmann fibers in the molecular layer, and other neuroglial cells in the granular layer and between blood vessels (Fig. 2 a-e). Rarely were labeled stellate or basket cells observed. There were two components of immunoreactivity in labeled cells—membranous and cytoplasmic. The membranous reactive material is detectable on the plasma membranes, on outer nuclear membranes but not in the nucleus itself, on outer mitochondrial membranes but not within mitochondria on the cristae, on outer membranes of the granular and smooth endoplasmic reticulum but not within the lumen, and on microtubules and neurofilaments. The cytoplasmic matrix appears as a dark reactive flocculent material between the cellular organelles. Where the label occurs in glial cells, the unlabeled neuronal elements are encircled by reactive processes, for example, around Purkinje cells (Fig. 2a), around synapses between Purkinje cells thorns and axonal boutons (Fig. 2 b and c), around mossy fiber rosettes and granule cells (Fig. 2d), and surrounding a blood capillary (Fig. 2e). No specifically labeled glial or neuronal elements were found in control tissue treated with cGMP IgG adsorbed with cGMP.

**DISCUSSION**

Immunocytochemical methods can be used to demonstrate the cellular and subcellular locations of cGMP immunoreactivity. These are to a large extent in neuroglial cells throughout the cerebellum but in particular those adjacent to and surrounding Purkinje cells. The intensity and distribution of cGMP immunoreactivity are affected by pharmacological manipulation and the cellular locations and levels of such changes can be studied. Substances that increase cerebellar activity increase cGMP immunoreactivity and substances that decrease cerebellar activity decrease cGMP immunoreactivity. Because the majority of cGMP is in neuroglial cells, it would be expected that their activity would fluctuate in response to activity of adjacent or related neurons. Although the data indicate that these substances can alter levels of cGMP detectable by immunocytochemical methods, their mechanisms of action remain obscure. Interpretation of the pharmacological data is impeded by the reduction in cerebellar activity imposed by the necessary anesthesia of the experimental animals. Moreover, it is not clear, for example, whether the increase in cGMP immunoreactivity after drug treatment is the direct result of drug action or selective neuronal activity or is secondary to other effects, such as interference with calcium influx or GABA release and uptake. The present study provides evidence for the presence of membranous (particulate) and cytoplasmic (soluble) cGMP, particularly in neuroglial cells, and indicates that cGMP is in a position to interact with externally applied substances, including putative neurotransmitters, as well as participate in the regulation intracellular processes. This focuses our attention on the possible nonneuronal, nonsynaptic functions of cGMP and forces us to recognize the importance of the neuroglia in neurotransmitter-related neuronal events.

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