Ultrastructural Localization of Cyclic GMP and Cyclic AMP in Rat Striatum

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ABSTRACT   The subcellular localization of cyclic GMP and cyclic AMP in the rat caudate-putamen has been studied using horseradish peroxidase immunocytochemistry. Both of the putative neurotransmitter second messengers were visualized in neurons and glial cells at light microscopic resolutions, but not all cells of either category gave detectable staining. This was confirmed at the ultrastructural level where both stained and unstained elements of the same cell type were found within the same field. A striking variation was seen in cyclic nucleotide staining intensity within individual neural and glial cells. Both of the cyclic nucleotides were detected within postsynaptic terminal boutons and within astroglial processes. Cyclic GMP postsynaptic staining was stronger than glial staining, whereas the localization pattern was reversed for cyclic AMP. The synaptic localization of cyclic AMP and cyclic GMP immunoreactivity adds support to the idea that these compounds have an influential role in synaptic function within the striatum.

A variety of evidence suggests that cyclic 3',5'-guanosine monophosphate (cGMP) and cyclic 3',5'-adenosine monophosphate (cAMP) are involved in the regulation of neural function. The evidence suggesting that cAMP functions as a second messenger in transducing specific neurotransmitter actions onto target cells has been extensively documented (6). Other studies suggest that cGMP is involved in physiological processes at synapses in both peripheral (14, 15) and central (8) nervous tissue. cGMP has been shown to accumulate in superior cervical ganglia in response to either electrical stimulation (18; McAfee, personal communication, 1981), or applied acetylcholine (13, 14, 23), and other depolarizing agents (9, 19). In the cerebellum, cGMP levels are increased by superfusing the tissue with the neurotransmitters norepinephrine, γ-aminobutyric acid, or glutamate (4, 5, 16, 17). A catecholamine-sensitive guanylate cyclase has recently been described in human caudate nucleus (10). A second line of evidence implicating cGMP in postsynaptic events has come from studies of cyclic nucleotide phosphodiesterase (PDE) (EC 3.1.4.17), the enzyme that hydrolyzes the cyclic nucleotides. These studies showed that the PDE, which is specific for cGMP and modulated by calcium and the calcium-dependent regulator, is concentrated at postsynaptic sites in the rat caudate-putamen complex as assessed cytochemically (2).

Although accumulation of both cAMP and cGMP is demonstrable after application of putative neurotransmitters and after synaptic activation, anatomic studies have not been extensively used to determine the location of these small chemicals in relation to their hypothesized site of activity as second messengers for primary neurotransmitters. Previous immunohistochemical studies have shown cGMP to be localized in neuroglia cells, and stellate and basket neurons in fresh and fixed cerebella (5), and in fibrillar processes and small neurogliaform cells in fresh-frozen caudate-putamen (3).

Apart from the isolated report of Chan-Palay and Palay (5), there has been no attempt to localize the cyclic nucleotides at the ultrastructural level and thereby determine the subcellular site of cyclic nucleotide accumulation in relation to other elements of the cellular milieu. We have now examined brain tissue, fixed by perfusion to give adequate ultrastructural preservation and subsequently processed with immunoperoxidase staining, to determine cyclic nucleotide localization. The results confirm that both cGMP and cAMP can be reproducibly localized to specific and different subcellular compartments in the same brain region.
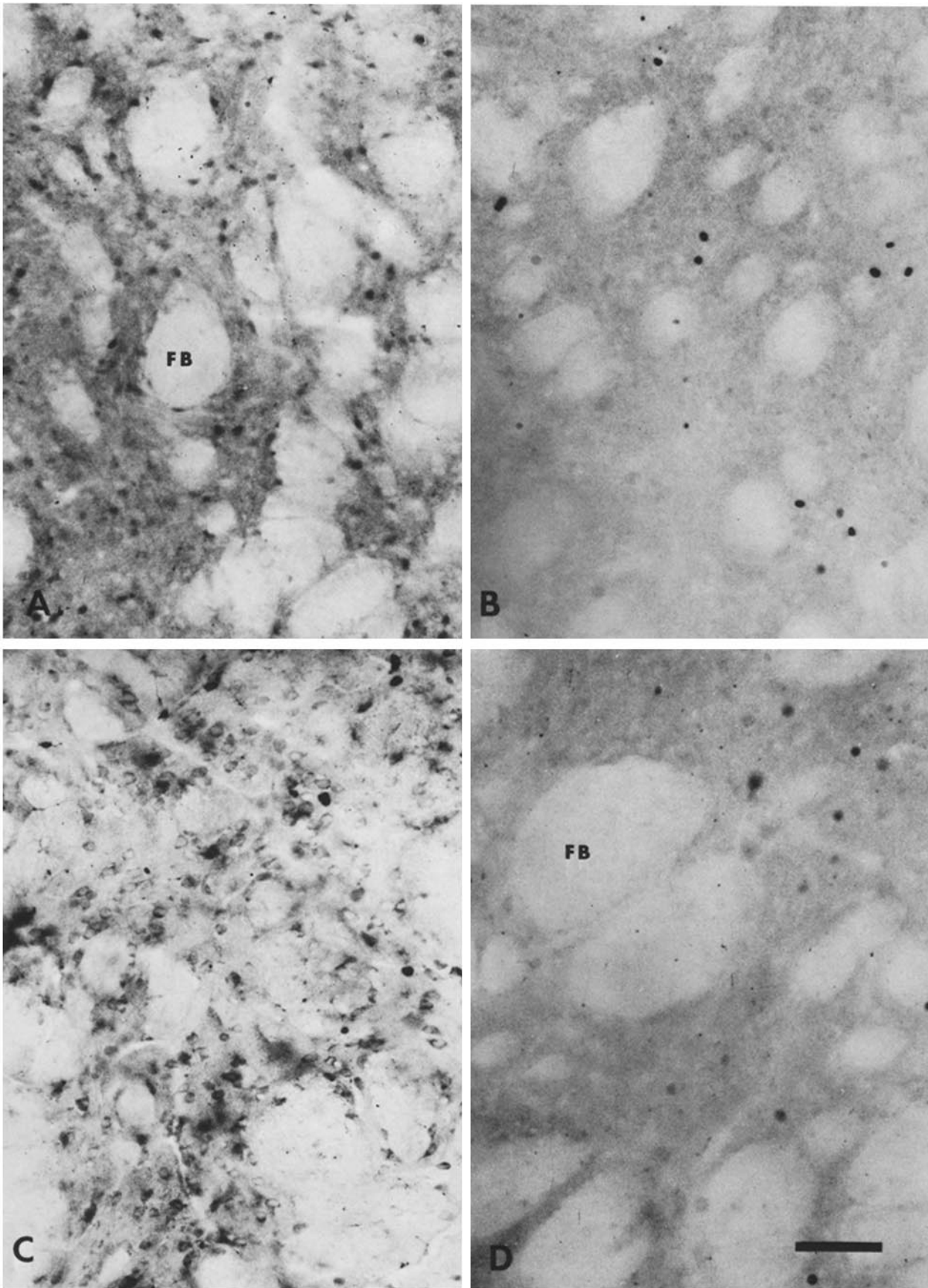
MATERIALS AND METHODS

Antibody Preparation

FIGURE 1   Cyclic nucleotide immunohistochemistry in rat striatum. 50-μm vibratome sections of aldehyde-fixed tissue were processed using horseradish peroxidase (PAP) immunochemistry. Bar, 50 μm. × 160. (A) Caudate nucleus after incubation with anti-cAMP antiserum. FB denotes a nonreactive fiber bundle perforating the neuropil. (B) Caudate nucleus following preadsorption of anti-cAMP antiserum with 1 mM cAMP. Note absence of PAP staining except in erythrocytes. (C) Striatum after incubation with anti-cGMP antiserum. Note pale nuclei of reactive cells. (D) Striatum after incubation of anti-cGMP antiserum with 1 mM cGMP. No staining is seen within neurons or glia.

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Immunocytochemistry Procedure

Sprague-Dawley rats, 200–300 g, were anesthetized with sodium pentobarbitol and transcardially perfused with 4% paraformaldehyde, 0.5% glutaraldehyde in 0.05 M sodium cacodylate buffer with 0.25 M sucrose, pH 7.4, at 4°C. The brain was removed and placed into fresh, cold fixative for 1 h. Slabs of the telencephalon were sectioned at 50 μm with a vibratome (Oxford Instruments, San Mateo, Calif.) and collected into PBS at 4°C. The coronal sections were incubated as free-floating pieces in the primary and secondary antisera diluted into PBS at room temperature. The antibodies against cAMP and cGMP were used at a 1:100 dilution. The incubation procedure was performed, as follows, with continuous, gentle agitation: (a) 1% normal goat serum (NGS; N. L. Cappel Laboratories, Inc., Cochranville, Pa.) in PBS for 30 min; (b) 1° (primary) antibody incubation for 30–60 min in PBS with 1% NGS; (c) PBS wash for 60 min; (d) goat-anti-rabbit IgG (Cappel Laboratories) at 1:50 dilution in PBS for 30–60 min; (e) PBS wash for 60 min; (f) rabbit peroxidase-antiperoxidase (Cappel Laboratories) at 1:50 dilution in PBS for 30 min; (g) PBS wash for 60 min; (h) 3,3-diaminobenzidine hydrochloride (Sigma Chemical Co.), developed with hydrogen peroxide for 10–15 min in PBS (pH 6). Tissues were washed for 60 min in PBS and osmicated, then dehydrated and infiltrated with either Araldite 502 or Spurr (22) resin. Thin sections were cut on a Reichert ultramicrotome and examined, unstained, in a Philips EM 400.

Light Microscopy

Tissues were processed, as described above, through vibratome sectioning. The 50-μm sections were rinsed thoroughly in PBS, then mounted onto chrome-alum-coated glass slides and cover-slipped using PBS:glycerin (1:9). Sections were examined with a Zeiss photomicroscope 3, and photographed with 35-mm black and white (Tri-X) or color (Ektachrome 400) film. The immunochemical staining was considered specific when horseradish peroxidase staining could be blocked by the addition of 1 mM cAMP or cGMP to an equal volume of the appropriate antibody for 1 h at 30°C, followed by continued incubation of the mixture at 4°C for 12 h. The blocked antisera were used at 1:100 dilutions. Another criteria for specificity was omission of the 1° cyclic nucleotide antibody from the procedure and elimination of stained tissue elements. Substitution of preimmunized rabbit serum for the 1° antibody was also performed, with subsequent lack of tissue immunoreactivity.

All other chemicals used were of reagent grade.

RESULTS

Immunohistochemical staining for cGMP and cAMP within the rat caudate-putamen complex demonstrated positively reacting neurons and glial cells. cAMP-stained tissue showed an abundance of 12-μm Diam neurons throughout the substance of the striatum (Fig. 1A). Glial cells were easily visualized bordering nonreactive fiber bundles (FB) which perforate the rodent caudate nucleus. Not all neurons or glial elements exhibited positive cAMP reactivity, as could be demonstrated by counterstaining with toluidine blue (data not shown). Preadsorption of the antisera with cAMP (see Materials and Methods) blocked all staining activity other than endogenous peroxidase within erythrocytes (Fig. 1B). Immunohistochemical specificity of the antisera was also examined after challenging each antibody with the opposite cyclic nucleotide. After incubation of anti cAMP antibody with cGMP (as described in Materials and Methods), cAMP localization was readily visualized and was virtually identical to the staining pattern seen in Fig. 1A.

cGMP immunohistochemical staining also showed neurons and glial cells to have antigenic sites for this cyclic nucleotide (Fig. 1C). The nuclei of the 8-to-12-μm neurons are generally free of peroxidase staining. Reactive glial cells were also seen bordering the fiber bundles perforating the neuropil of the striatum. Fig. 1D demonstrates the staining pattern after preadsorption of the antisera with 1 mM cGMP (see Materials and Methods). The only peroxidase reaction product is due to endogenous activity, especially within erythrocytes. After incubation of anti cGMP antibody with cAMP, immunohistochemical analysis demonstrated PAP-reaction product deposition comparable to that seen in Fig. 1C. The improved morphology obtained with aldehyde-fixed tissue allowed visualization of substantial lengths of proximal dendrites in the cGMP-
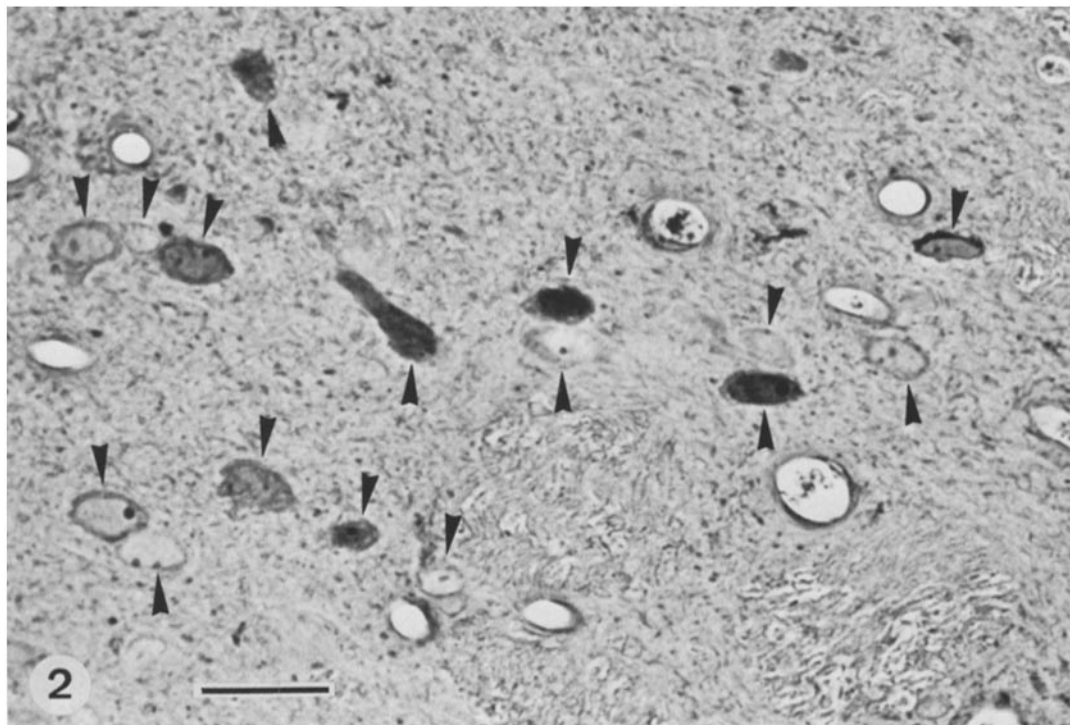

FIGURE 2   1-μm plastic-embedded section of caudate nucleus following incubation with anti-cGMP antiserum and postfixation with osmium tetroxide. All cell bodies and parts of cell bodies in the field are indicated with arrowheads. Staining intensity varies from strongly positive to no detectable reaction. The neuropil is filled with punctate-stained sites. Bar, 50 μm. × 330.

stained material (Fig. 2). The pale nuclei of the positively reacting neurons are easily seen and exhibit prominent nucleoli. Nonreactive cells can also be located in this semithick 1-μm section after toluidine blue counterstain.

Ultrastructural localization of cGMP and cAMP, using immunocytochemistry, demonstrated differences in the subcellular organelles retaining antigenicity to these two chemicals. cAMP immunoreactivity could be seen in neurons and glial cells of the caudate-putamen. The prevalent intracellular staining for this nucleotide was in the cytoplasm of astroglial processes and within postsynaptic terminal boutons (Fig. 3). The immunocytochemical localization of cGMP could also be found within both neurons and glial elements (Fig. 4). The predominant subcellular structure associated with the accumulation of this cyclic nucleotide was the postsynaptic terminal, especially the subsynaptic density of type-1 axospinous contacts (Fig. 4, inset). Nonreacting type-1 synapses were also seen within the tissue, and all type-2 synapses lacked cGMP as well as cAMP immunoreactivity. Heavily stained ribosome rosettes in primary dendrites could also be detected after cAMP or cGMP immunocytochemistry procedures.

DISCUSSION

The pattern of cGMP and cAMP staining in aldehyde-fixed striatum agrees with the visualization of fluorescein-labeled antibodies against these compounds as seen in fresh-frozen caudate-putamen (3). Both cyclic nucleotides are reproducibly seen within neurons and glial cells of the striatal neuropil. Fixation improves the morphology of cellular structures containing immunoreactivity for cGMP and cAMP, and allows identification of positively reacting tissue elements not previously seen. Further, ultrastructural deposition of horseradish peroxidase reaction product allows determination of the subcellular sites where the cyclic nucleotides accumulate.

In light microscopic preparations it is clear that not all neurons or glial cells are positively reactive for the cyclic nucleotides. This finding was confirmed at the electron micro-
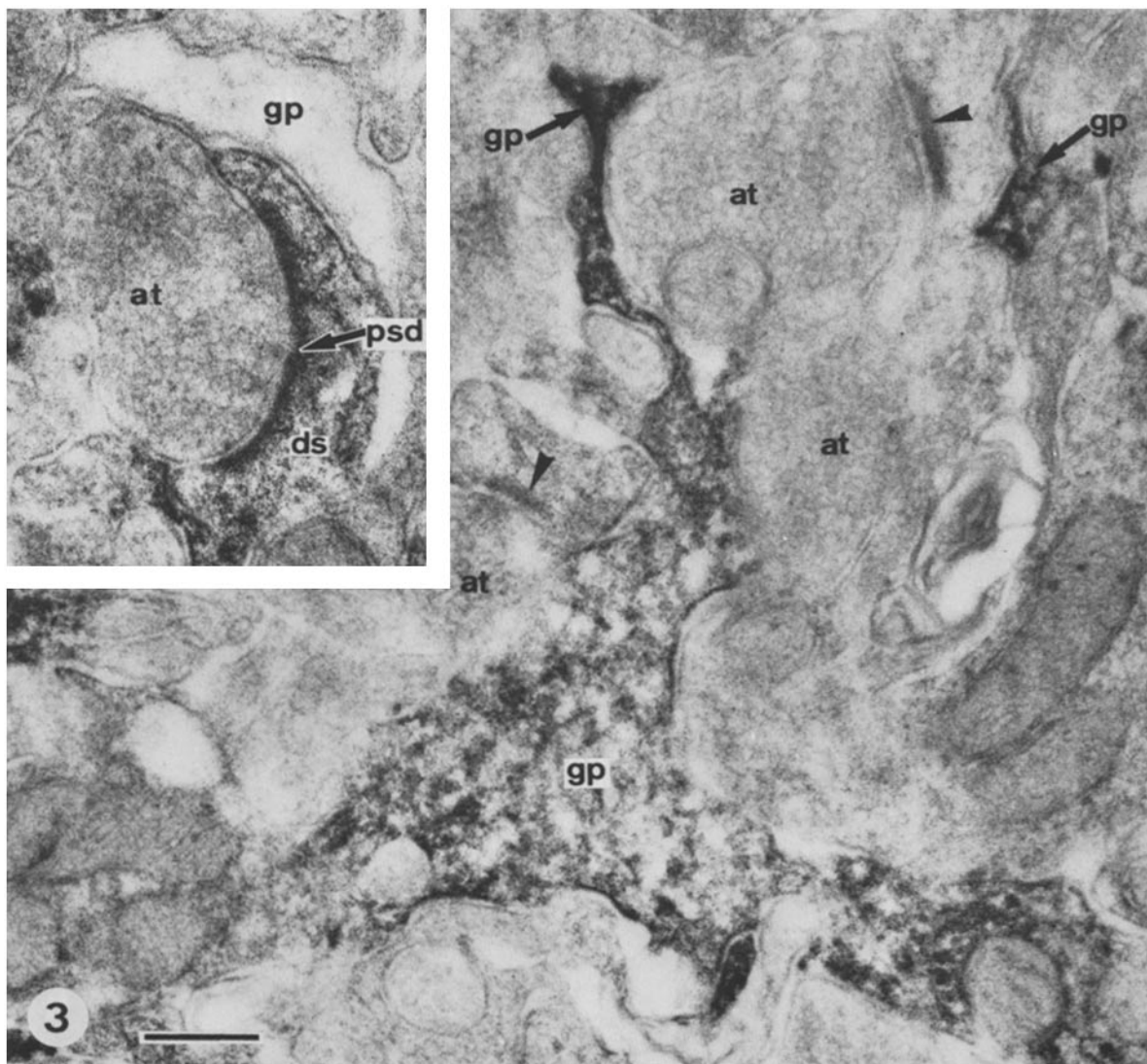

FIGURE 3   Ultrathin section of caudate nucleus after staining with anti-cAMP antiserum, osmium tetroxide postfixation, and embedding in plastic. An extensive stained glial process (gp) permeates the neuropil. A small process is also marked. Three axon terminals (at) show no PAP reaction, nor do components of the synaptic junctions (arrowheads). The inset shows an axospinous type-1 synapse from the same section in which the dendritic spine cytoplasm (ds) is stained and the postsynaptic density (psd) is more strongly reactive. Neither the axon terminal nor the adjacent glial process is reactive. Bar, 0.25 μm. × 60,000.
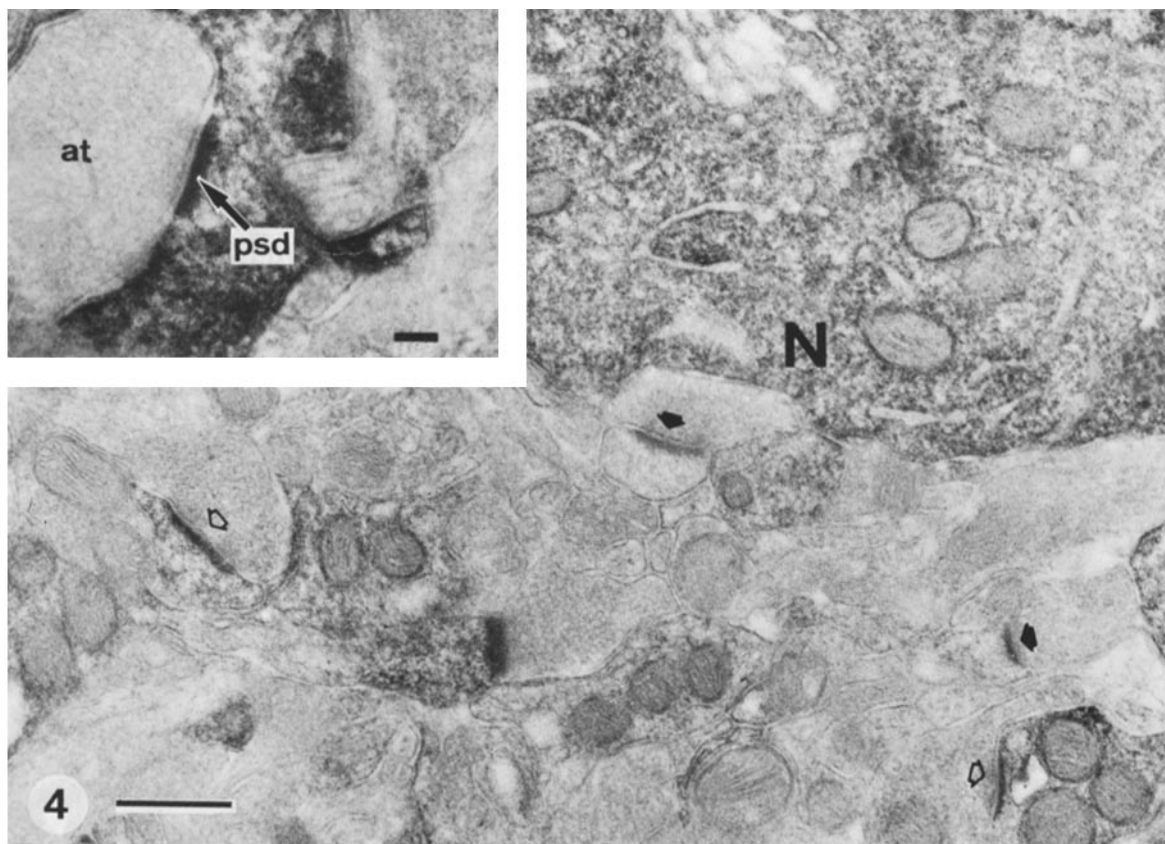
FIGURE 4 Caudate nucleus after incubation with anti-cGMP antiserum, osmium tetroxide postfixation, and embedding in plastic. Main field shows a positive PAP reaction in the cytoplasm of a neuron (N). Solid arrows point to nonreactive synapses. Open arrows denote cGMP-immunoreactive terminals. Note lack of reaction product in the presynaptic terminal (at) of the inset, and the strong reaction in the postsynaptic density (psd). Bars, main field 500 nm, inset 100 nm. Main field × 30,000, inset × 56,000.

scopic level of resolution where both stained and nonreactive glial and neuronal elements are found contiguous to one another. Such marked differences in antigenic immunoreactivity are unlikely to have arisen because of variations in antibody penetration because these differences are found even between adjacent elements of the same cell type. Furthermore, within the same small field of <10 μm^2, we can observe various degrees of peroxidase reaction in different tissue elements. Staining is commonly at the lowest level of certain recognition in cell bodies, intermediate in positively reacting postsynaptic terminals, and highest in astroglial processes adjacent to synapses. Graded responses in cyclic nucleotide staining suggest that different levels of cAMP and cGMP can be demonstrated by the peroxidase technique even in electron microscope preparations. The existence of graded reactivity also suggests that lack of staining in some cells and processes is genuine and presumably occurs because they contain levels of cyclic nucleotide below the limits of detectability of the immunoperoxidase method.

Similarities and differences in the distribution pattern of the two cyclic nucleotides are apparent at the ultrastructural level. Both cAMP and cGMP accumulate in postsynaptic elements of axospinous synapses. This localization agrees with a variety of data suggesting that the rate of synthesis of these molecules is regulated, at least in part, by postsynaptic transmitter receptors (4, 9, 12, 13, 16, 18, 23). It also coincides with the major sites of both cAMP and cGMP phosphodiesterase activities demonstrated cytochemically within the striatum (1, 2).

cGMP staining was reproducibly more intense at postsyn-

aptic terminals than in astroglial cells surrounding the boutons. The finding of high concentrations of cGMP at axospinous postsynaptic sites in this study, together with our previous visualization (2) of high cGMP phosphodiesterase activity at morphologically equivalent sites, places two primary components of the cGMP system at the expected position for this cyclic nucleotide to participate in synaptic transmission within the striatum. This pattern of staining intensity was reversed for cAMP immunoreactivity, e.g., staining was more prevalent in glial processes. This finding correlates well with evidence suggesting that the cAMP response seen in brain slices following application of norepinephrine (6) may be more closely associated with glial cells than with neurons (11, 20). The presence of cAMP in some presynaptic elements may be correlated with observations that cAMP has effects on presynaptic transmitter release of cholinergic (12) as well as noradrenergic (24) synapses.

Perhaps the most striking thing to emerge from this study is the apparent adjacent existence of cells and processes whose levels of cAMP or cGMP staining vary from a dense deposit of reaction product to no detectible staining reaction. Do such cells differ constitutively in their basal levels of these cyclic nucleotides, or do they represent widely different levels of activation of cells that are fundamentally similar in their capacity to metabolize these chemicals? Our ability to visualize graded staining reactions in both light and electron microscope preparations will allow us to conduct experiments in which the metabolic state of caudate cells is manipulated by stimulation and/or interruption of input pathways, and to determine

whether graded responses occur in the various cell types giving basal level staining or whether there is an increase in the population of stainable cells.

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