Somatostatin Immunocytochemistry in the Rabbit Retina

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In previous work, radioimmunooassay was used to document the presence of somatostatin-like immunoreactive material in the rabbit retina. The present study was undertaken to determine the cellular localization of that material by light microscopic immunocytochemistry. Rabbit retinas were fixed by immersion in paraformaldehyde-lysine-periodate and reacted, either as whole retinas or as 50 μm Vibratome cross-sections, with an antiserum directed against somatostatin-14. Consistent staining of neuronal perikarya was seen only in the retinas of rabbits that had been pretreated with intravitreal injections of colchicine. Specifically stained cell bodies are present in the ganglion cell layer; the cells give rise to fibers in both the innermost and outermost sublaminae of the inner plexiform layer. In retinal whole mounts, the cells possess two or three primary dendrites with sparse branching. The dendritic fields are up to 1 mm in diameter, and adjacent dendritic fields overlap. Many cells have a thin varicose process arising from the soma or a proximal primary dendrite; these processes branch repeatedly within the retina and resemble intraretinal axons. The somatostatin-reactive cells may be associative ganglion cells or displaced amacrine cells; it is less likely that they are ganglion cells with axons projecting to the brain. Invest Ophthalmol Vis Sci 27:316-322, 1986

The functions served by neuropeptides in the mammalian central nervous system are still largely unknown. In experimental systems, peptides have been found to act as conventional neurotransmitters by exerting direct effects on post-synaptic membranes, to act at some distance from the site of release, and to interact with the release and action of other neurotransmitters (see Ref. 1 for review). The retina is a particularly attractive model system for the study of central nervous system neuropeptide action. A variety of peptides are present in the retina, and an extensive literature correlates cell shape and neurotransmitter content (see Ref. 2 for review). In an effort to learn about the functional role of somatostatin, we have employed immunocytochemical methods to explore the shape of the cells containing that peptide in the rabbit retina and have compared their cellular morphology with the classes of neurons known to exist in the retina.

Somatostatin-like immunoreactive material (SLI) is present in the retinas of many vertebrates, including frog, chicken, pigeon, goldfish, cow, rat, human, and rabbit. The majority of the SLI in the rabbit retina behaves chromatographically as somatostatin-14, with 15-20% of the SLI being somatostatin-28-like material. We have employed selective neurotoxins in an effort to determine the cell type(s) which contain SLI. The intraocular injection of the monoamine neurotoxins 6-hydroxydopamine and 5,7-dihydroxytryptamine has no effect on retinal concentration of SLI, implying that SLI in the rabbit retina is not present in dopamine-containing or indolamine-accumulating neurons. However, the intraocular injection of kainic acid at doses sufficient to produce greater than 90% reduction in retinal dopamine and immunoreactive substance P content reduces the total retinal content of SLI by only about 50%. This finding suggests that two approximately equal pools of SLI may be present in the rabbit retina, one sensitive to and one resistant to the neurotoxic actions of kainic acid. These separate pools may correspond to two SLI-containing cell types in rabbit retina.

Materials and Methods

Animals

The investigators were governed by the principles of the ARVO Resolution on the Use of Animals in Research. Male, Dutch belted rabbits weighing 1.5-2.0
kg were maintained on a 10 hr:14 hr, light:dark schedule with free access to food and water. For all intravenous injections, the rabbits were sedated with intravenous pentobarbital (20 mg/kg), and the eyes were topical anesthetized with 1% proparacaine. In some animals, colchicine (50–100 μg in 50 μl normal saline) was injected intravitreally into one or both eyes through a 30-g needle 40 hr prior to death. Some rabbits received kainic acid, 120 nmol (25 μg) in 50 μl normal saline, injected intravitreally into one eye 1 wk prior to death; 50 μl saline was injected into the other eye 1 wk prior to death; 50 μl saline was injected into the other eye as a control. Rabbits were killed with an intravenous injection of pentobarbital (80 mg/kg).

**Immunocytochemistry**

Posterior eye cups, with vitreous removed, were immersed for 4–12 hr at 4°C in 2% paraformaldehyde, 0.1 M L-lysine, 0.01 M sodium m-periodate, 0.05 M sodium phosphate buffer, pH 7.4. The eye cups were washed in 0.1 M sodium phosphate buffer, pH 7.4, and the retinas were dissected from the pigmented epithelium. Retinas were then processed for immunocytochemistry either as whole retinas or were cut into sectors, embedded in agarose (Type VII, Sigma Chemical Co.; St. Louis, MO), and sectioned at 50 μm on a Vibratome.

All immunocytochemical reagents were diluted in 0.1 M sodium phosphate buffer, pH 7.4. Tissue was incubated in 10% normal goat serum containing 0.2% or 0.5% Triton X-100 for 30–60 min and then with primary antiserum (overnight at 4°C for retinal cross-section and 5 days at 4°C for whole retinas).

Antiserum to somatostatin-14 was obtained from Immunonuclear Corporation (Stillwater, MN; Lot #27092) and was used at a dilution of 1:2,000 in the presence of 0.2% Triton X-100 for cross-sections and 0.5% Triton X-100 for whole retinas. Specificity of the staining procedure was demonstrated by abolition of cell body or fiber staining with the inclusion of somatostatin-14 in the incubation with primary antiserum. For cross-sections, 10 ng/ml somatostatin-14 was included; and for whole retinas, 100 ng/ml. We have verified the specificity of this antiserum in a radioimmunoassay system. Sites of antibody binding were visualized with the ABC (avidin-biotin-peroxidase) method (Vector Laboratories; Burlingame, CA) using diaminobenzidine (Sigma Chemical Company) as the chromagen.

Retinal cross-sections were mounted in Protex, and whole retinas were mounted in buffered glycerol. They were examined by bright field, phase contrast, and Nomarski optics. Cell body area was determined in whole mounts by making a camera lucida drawing of the perikaryon and measuring the area with a sonic digitizer.

**Radioimmunoassay**

Retinas were dissected, extracted in 2 M acetic acid, and assayed by a specific double antibody radioimmunoassay as described previously. Protein was assayed by a fluorometric procedure.

**Results**

In cross-sections of rabbit retinas pretreated with colchicine, specifically stained cell bodies are present in the ganglion cell layer (GCL) (Figs. 1B, C). Of hundreds of stained cells, only one lies in the inner nuclear layer. Immunostained varicose fibers occupy both the innermost (sublamina 5) and the outermost (sublamina 1) sublaminas of the inner plexiform layer (IPL) (Figs. 1A, B). The fibers in sublamina 5 are often continuous with immunostained cell bodies (Fig. 1C).

In whole mounts, immunostained cells were seen only in retinas pretreated with colchicine, and then only in the GCL (Fig. 2). The cells are relatively sparse and are evenly distributed throughout the retina inferior to the medullary rays. No stained cell body was identified superior to the myelinated fiber bundle. There was no apparent difference in the density of stained cell bodies between central and peripheral regions. The cells are oval or polygonal. In the mid-periphery of a fixed retina mounted in glycerol, the mean area of the cell bodies was 92.6 ± 3.6 μm² (mean ± S.E.M., n = 22).

The cells each give off 2 or 3 primary dendrite-like processes in sublamina 5 of the IPL; these dendrites branch sparsely (Figs. 2, 3). The processes can be followed for distances up to 500 μm in whole mounts, and the dendritic fields of adjacent cells overlap extensively (Fig. 4). The dendrites can frequently be followed as they cross the IPL 100–400 μm from the cell body and course in sublamina 1 (Figs. 3, 4). Most immunostained processes are varicose, have occasional spine-like appendages and are often found in juxtaposition to one another (Fig. 5). In many cells, a single, thin, varicose process arises from the cell body or a proximal dendrite. These processes branch repeatedly (Fig. 3). No immunostained cells, but many immunostained fibers, were seen dorsal to the myelinated fiber bundle in both colchicine pretreated and untreated retinas.

When retinas of kainic acid-treated rabbits were examined immunocytochemically, no stained fibers or cell bodies were present even with colchicine pretreatment. The saline-treated retinas from the same animals, however, were identical to those described above.

We considered the possibility that the 50% depletion of SLI measured by radioimmunoassay in our previous studies with kainic acid might be explained by the nonuniform distribution of SLI-containing cells in the retina combined with a failure of the intracellular neu-
rototoxin to reach equal concentrations in dorsal and ventral retina. The effect of kainic acid on SLI content, determined by radioimmunoassay, was therefore measured in the dorsal and ventral retina separately.

Six rabbits had 120 nmol of the neurotoxin kainic acid injected into one eye and saline injected into the opposite eye as a control. One week later the animals were killed and the retinas were removed. Each retina was cut horizontally just inferior to the myelinated fiber bundle, dividing the retinas into two unequal sections, a dorsal section corresponding to that region in which no immunostained cell bodies were seen and a ventral section where the cell bodies containing SLI were consistently seen. In the dorsal and ventral sections of the kainic acid treated retinas, the mean content of SLI was reduced to 53 ± 7% and 61 ± 11%, respectively, of the content of the control retina of the same animal. For comparison, substance P content was reduced to 10 ± 3% of control in the dorsal retina and 6 ± 1% of control in the ventral retina. These observations confirm our previous measurements of SLI depletion by kainic acid, but do not suggest a dorsal-ventral gradient in the action of the neurotoxin.

**Discussion**

Retinal neurons containing SLI have been identified with immunocytochemical techniques in the
Fig. 2. Two somatostatin-reactive cell bodies in the ganglion cell layer as seen in flat mounts. The immunostained cells are bi- or tripolar, but there is no consistent orientation of the cell axis. (Calibration bar = 100 µm).

In these species, the predominant localization of SLI-containing perikarya is in the INL; in fish, rat and human there are cells in the GCL in addition. In the rabbit retina, however, the only immunostained cells seen, with but a solitary exception among all of the cells examined, were in the GCL. Since this was true whether whole retinas or retinal cross-sections were processed for immunocytochemistry, this finding is not an artifact of antibody penetration into the tissue.

The SLI-containing cells were observed only inferior to the medullary rays, but are relatively evenly distributed throughout the inferior retina. It is possible that this unusual distribution is an experimental artifact and that the colchicine, which is essential for the visualization of the SLI-containing cell bodies, failed to
Fig. 3. Camera lucida drawing of a single SLI-containing neuron. The cell body lies in the GCL and the proximal dendrites in sublamina 5 of the IPL. At the double arrows, the distal segments of 2 processes cross the IPL; the dotted portion of the processes are in sublamina 1. In most of the cells studied, dendrites cross the IPL in their distal segments. A thin, varicose process also arises from the cell body (open arrow); it is possible that this is an axon. (Calibration bar = 250 μm).

reach the dorsal retina. We attempted to deal with this possibility by injecting the colchicine from superior, medial, and lateral quadrants of the eye in different animals and by aiming the needle so as to be sure to inject the colchicine near the superior retina. In no instance, however, did we stain a single cell body superior to the myelinated fiber bundles.

We cannot be certain whether the stained cells are

Fig. 4. Camera lucida drawing of 4 cells containing SLI in the central retina below the medullary rays. The distances between cell perikarya and the density of the plexus formed by their processes are representative of the entire ventral retina. (Calibration bar = 250 μm).

Fig. 5. A. Immunostained process with spine-like short appendages (arrows). These spine-like processes are a consistent feature of most of the stained dendrites. B. Juxtaposed segments of processes arising from different cells. This high power micrograph is of an area on the left side of the camera lucida representation in Figure 4. The distal extent of stained processes at times appears to terminate in apposition to another stained process in the same sublamina. (Calibration bars = 50 μm).
ganglion cells or displaced amacrine cells. The cell body size, with a mean area of 92.6 μm² in flat mounts, is in the region of overlap between ganglion cells and displaced amacrine cells of the rabbit.28 The morphology of the SLI-reactive cells does not correspond to any of the four ganglion cell types described in the rabbit,29 and we cannot unequivocally identify axons belonging to these cells. No immunostained fibers were seen in the nerve fiber layer either with or without colchicine treatment. However, many cells examined in flat mounts (Fig. 3, for example) had a thin varicose process arising from the cell body or proximal primary dendrite. These processes frequently branched at right angles within the retina and could represent locally arborizing axons. Therefore, it is possible that the SLI-containing cells are associational ganglion cells.30

If the SLI-containing cells are ganglion cells which project to brain, one would expect to detect SLI in the optic nerve. Radioimmunoassay of the optic nerve, however, fails to demonstrate SLI (unpublished). We have not examined optic nerve by immunocytochemistry. A detailed mapping, either by radioimmunoassay or immunocytochemistry, of SLI distribution in central visual system nuclei of the rabbit, including potential sites of termination of retinal afferents, has not been reported.

The observation that colchicine allows the detection of SLI in cell bodies is of interest. Generally, colchicine is thought to improve the immunocytochemical visualization of neuropeptides in cell bodies by blocking axonal transport and allowing newly synthesized peptide to accumulate in the perikaryon. If this is the mechanism, the positive effect of colchicine in the rabbit retina suggests that the SLI-containing cells indeed possess axons. This line of reasoning is highly speculative, however, as colchicine may affect cellular processes other than axonal transport.

On the basis of cell counting data, it has been estimated that cholinergic displaced amacrine cells account for at least 85% of the displaced amacrine cells of the rabbit.31 To date, no other type of displaced amacrine cell has been described in that species. The SLI-containing cells are morphologically distinct from the cholinergic cells; they are larger, on average, have wider dendritic fields, and have fewer primary dendrites. Furthermore, the SLI-containing cells give rise to processes in both inner and outer sublaminas of the IPL, whereas the cholinergic displaced amacrine cells give rise to processes only in the innermost sublamina of the IPL. These observations suggest that the SLI-containing cells do not form a subgroup of cholinergic displaced amacrine cells but instead form a previously undescribed group of cells of the rabbit retina.

A major limitation of immunocytochemical methods is that one can never be certain that the techniques employed are sufficiently sensitive to detect all somatostatin-containing structures. In this study, we detected stained cell bodies only if retinas were pretreated with colchicine. Therefore, the technique is insufficiently sensitive to detect all SLI-containing cells under normal conditions. When kainic acid was administered, even with colchicine pretreatment, no immunostained cell bodies were found. However, radioimmunoassay of kainic acid treated retinas, processed in experiments run in parallel with the immunocytochemistry, demonstrated persistence of about 50% of the SLI.

Therefore, in both normal and kainic acid treated retinas, there are SLI-containing elements which escape detection by our immunocytochemical procedures. One possibility is that there is a single SLI-containing cell type, but we can visualize only a fraction of that population. Alternatively, there may be SLI-containing elements different than those shown here. In analogy with other species, a prime candidate for such unstained structures is amacrine cells of the INL. A more speculative hypothesis would be that SLI is contained within horizontal, bipolar, or photoreceptor cells, all of which are relatively resistant to kainic acid, but which have not been shown to contain SLI in any mammal. Even more speculative possibilities are Müller cells or afferent terminals and fibers from the brain. We have no direct data pertaining to any of these possibilities, but we have excluded obvious technical artifacts.

The SLI-containing cells possess large, overlapping dendritic fields. Their processes cover the ventral retina with a loose meshwork of SLI-containing fibers. These large dendritic fields suggest that the cells are not involved in pattern vision, but they are well positioned to be strongly influenced by overall environmental light levels. The only other population of retinal cells known to have a similar distribution are the ganglion cells of the goldfish that project to the suprachiasmatic nucleus.32 These cells are presumably involved in regulating circadian rhythms of the teleost. The function of the SLI-containing cells of the rabbit is as yet unknown.

Key words: somatostatin, retina, rabbit, immunocytochemistry, ganglion cells

Acknowledgments

The technical assistance of Ms. Linda Lorenz, Ms. Susan Murchison, and Mr. Lawrence Cherkas, and the secretarial assistance of Ms. Kathleen Kreatz and Ms. Eleanor Bell are
References