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Immunoreactive and biologically active somatostatin-like material in rat retina

(growth hormone release-inhibiting hormone/somatotropin release-inhibiting factor/neuropeptides/retinal degeneration)

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ABSTRACT **Somatostatin-like activity, as determined by radioimmunoassay and bioassay, is present in HCl extracts of rat retina. The concentrations of immunoreactive somatostatin are 612 ± 43 (mean ± SEM) pg per whole retina or 0.621 ± 0.044 pg/μg of protein in retinas from rats killed by decapitation, values which are not significantly different from those in retinas from rats killed by ether inhalation. The immunoreactive somatostatin was partially purified and concentrated by immunoaffinity chromatography. Both the crude retinal extracts and the immunoaffinity-purified immunoreactive somatostatin inhibited, in a dose-related manner, the release of rat growth hormone from dispersed rat anterior pituitary cells in culture. When the immunoaffinity-purified immunoreactive somatostatin was subjected to gel filtration chromatography, 96% of the recovered somatostatin eluted as a peak corresponding in position to that of synthetic somatostatin. Retinas from a group of rats with hereditary degeneration of the photoreceptor cells and another group of rats studied 1 year after transection of the optic nerves demonstrated an increased concentration of immunoreactive somatostatin compared to controls.**

Somatostatin is a tetradecapeptide that was first isolated from sheep hypothalami as a consequence of its ability to inhibit growth hormone release from anterior pituitary cells (1). Subsequent studies using radioimmunoassay (RIA), bioassay, and immunohistochemical methods demonstrated a widespread distribution of somatostatin-like material in the nervous system, pancreas, and gut of many species (2). The additional fact that exogenously administered somatostatin perturbs a number of experimental models of neuronal activity provoked interest in the question of what, if any, physiological role somatostatin plays in the nervous system (3). For instance, somatostatin may function in the relay or modulation of peripheral sensory inputs to brain, as shown by its demonstration by immunohistochemistry in somatosensory primary neurons (4).

In this report we present evidence that immunoassayable and bioactive somatostatin-like material is present in the rat retina. A portion of our study has appeared in abstract form (5).

MATERIALS AND METHODS

Chemicals. Somatostatin (AY 24,910) used for immunization was obtained from Ayerst Laboratories (Montreal, PQ, Canada). Somatostatin (14-206-60) for use as a RIA standard and [Tyr^1]-somatostatin (35-242-30) were synthesized by J. Rivier. Na^{125}I (100 μCi/ml, 1 Ci = 3.7 × 10^{10} becquerels) was purchased from Amersham. Human serum albumin (Cohn fraction V) and bovine serum albumin were purchased from Sigma. Sephadex and Sepharose were obtained from Pharmacia. Materials for the RIA of rat growth hormone were obtained from the National Institute of Arthritis, Metabolism and Digestive Diseases (Bethesda, MD). The sources of the following peptides were: glucagon, gastrin, secretin, and gastric inhibitory polypeptide (J. Dupré); porcine insulin (Connaught Laboratories, Toronto, ON, Canada); substance P (S. Leeman); oxytocin (Peninsula Laboratories, San Carlos, CA); vasopressin and rat neurophysin 1 (M. Brownstein and H. Gainer); melanocyte-stimulating hormone inhibiting factor (Ayerst, lot AY 24,192); thyrotropin-releasing hormone and luteinizing hormone-releasing hormone (J. Rivier, lots 21-34-95 and 14-136-30, respectively); and rat growth hormone, rat luteinizing hormone, rat prolactin, and rat thyroid-stimulating hormone (National Institute of Arthritis, Metabolism and Digestive Diseases, respective reference preparations GH-RP-1, LH-13, PRL-RP-1, and TSH-RP-1).

Collection and Extraction of Tissue. Male Charles River (CD) rats were housed in a temperature- and humidity-controlled room with lights on from 0600 to 1800 hr and were allowed free access to food and drinking water. Rats that were used for the quantitative determination of immunoreactive somatostatin (IRS) content per individual retinal fragment ranged in weight from 200 to 250 g and were killed by decapitation or ether inhalation between 0930 and 1130 hr. The pools of retinal tissue used for the chromatographic studies of IRS were obtained predominantly from rats weighing 200–250 g, but also included retinas from rats weighing up to 600 g, and were collected at various times between 0830 and 1600 hr. The eyes were removed after death, the intact retinas were dissected free under visualization with a dissecting microscope, and each retinal fragment was placed in 0.5 ml of 0.1 M HCl. Dissection of the intact retina resulted in a tissue fragment that included a superficial portion of the ciliary body adherent to the retina. Microscopic study of hematoxylin/eosin-stained cross sections of the retinal tissues indicated that the plane of dissection extended between the photoreceptor cell and the choroid layers. This confirmed that all cellular layers of the retina were included in the tissue fragment. The fragments were homogenized with a glass vessel and rotating Teflon pestle with a clearance of 0.08–0.13 mm. The homogenate was frozen at −20°C overnight; after it was thawed at room temperature, it was centrifuged at 2000 × *g* at 4°C for 30 min. The clear supernatant was collected and stored at −20°C. The protein content of retinal extracts was determined by a fluorometric method using the reaction of the extracts with fluorescamine (6). Bovine serum albumin served as the reference protein.

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Abbreviations: IRS, immunoreactive somatostatin; RIA, radioimmunoassay; PETH, pink-eyed, tan-hooded (rats).

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Radioimmunoassay. An antiserum was used that had been raised in an adult male sheep against synthetic somatostatin by a technique that had previously proven succcessful in our laboratory for raising an antisomatostatin serum in rabbits (7). [Tyr^1]somatostatin was radioiodinated by a modification of the chloramine-T method of Greenwood *et al.* (8), and the radioiodinated product was purified by ion-exchange chromatography on carboxymethyl-cellulose as described (7). RIA was conducted with a 1/20,000 final dilution of antiserum and approximately 12,000 cpm of ^{125}I-labeled [Tyr^1]somatostatin in a final volume of 0.4 ml with an assay buffer of 0.1 M sodium phosphate (pH 7.2) containing 10 mM Na_2EDTA, 50 mM NaCl, 0.02% sodium azide, and 0.1% human serum albumin. The standard displacement curve was derived by using amounts of somatostatin ranging from 5 to 640 pg per assay tube. Prior to RIA, retinal extracts were neutralized with NaOH, with phenol red as a pH indicator. The neutralized extracts were centrifuged at 2000 × *g* at 4°C for 10 min to remove a small amount of precipitate that formed on neutralization. The supernatant was used for RIA. After an incubation period of 20–24 hr at 4°C, an appropriately diluted portion of rabbit anti-sheep serum was added to the RIA tubes. After incubation for an additional 16–20 hr at 4°C, RIA tubes were centrifuged and the radioactivity in the pellets was determined.

The minimum detectable concentration of the RIA, defined as the concentration of somatostatin that resulted in a binding of ^{125}I-labeled [Tyr^1]somatostatin to antiserum that was 2 standard deviations below the mean binding achieved in the absence of somatostatin, was 14.3 pg/tube (mean of 16 assays). The within-assay coefficients of variation determined for samples containing means of 110 and 304 pg of somatostatin per tube were, respectively, 13.9 and 12.3 (9 determinations). The between-assay coefficients of variation of samples containing means of 284, 144, and 75 pg of somatostatin per tube were 11.5, 13.2, and 13.7, respectively (16 assays). The following peptides in concentrations (mass/vol) of at least 100 times the maximum concentration of somatostatin used for the standard curve did not displace ^{125}I-labeled [Tyr^1]somatostatin from the sheep antiserum: gastric inhibitory polypeptide, gastrin, glucagon, insulin, luteinizing hormone-releasing hormone, melanocyte-stimulating hormone inhibiting factor, rat neurophysin 1, oxytocin, rat growth hormone, rat luteinizing hormone, rat prolactin, rat thyroid-stimulating hormone, secretin, substance P, and vasopressin. Studies using analogs of somatostatin have demonstrated that the antiserum recognizes the central portion of the somatostatin molecule, including the sequence of amino acids 4–10 (J. Epelbaum, P. Brazeau, and J. B. Martin, unpublished data).

The recovery of synthetic somatostatin added to eight separate retinal extracts was 105 ± 10% (mean ± SEM), indicating that no loss of immunological activity of somatostatin occurred before or during the RIA.

Dispersed Rat Anterior Pituitary Cell Bioassay. Dispersed rat anterior pituitary cell cultures were prepared by the method of Vale *et al.* (9) with omission of the resuspension of cells in Viokase. After a preincubation period of 3 days in 35 × 10 mm dishes (Corning) containing 2 ml of culture medium, the cells were washed once with culture medium. To 800 μl of fresh culture medium per dish was added 200 μl of either (*i*) a standard solution containing 20–1280 pg of somatostatin in 10 mM sodium phosphate buffer, pH 7.4/0.1 M NaCl/0.1% human serum albumin or (*ii*) a neutralized extract of retinas or a portion of the retinal IRS concentrated by affinity chromatography. Both experimental samples were diluted in the same buffer as somatostatin. After incubation for 3 hr, the culture medium was aspirated and stored at −20°C for subsequent assay of rat growth hormone by a previously described RIA (10).

Immunoaffinity Chromatography. An immunoglobulin fraction of the antisomatostatin serum was prepared by dropwise addition of a saturated solution of $(NH_4)_2SO_4$, adjusted to pH 6.8 with NaOH, to the antiserum at 4°C to yield a final concentration of 40% (vol/vol) saturated $(NH_4)_2SO_4$. The precipitate was collected by centrifugation at 12,000 × *g* at 4°C for 20 min, dialyzed for 2 days against a thrice replenished 10 mM sodium phosphate buffer, pH 7.4/0.15 M NaCl, and stored at −20°C in the above buffer.

The immunoglobulin obtained from 0.5 ml of antiserum was coupled to 4 g of cyanogen bromide-activated Sepharose 4B by incubation for 2 hr at room temperature in a 0.1 M $NaHCO_3$ buffer, pH 8.0/0.5 M NaCl with continuous end-over-end mixing. After the gel was washed on a Buchner funnel with the $NaHCO_3$ buffer, it was incubated with 1 M ethanolamine dissolved in the $NaHCO_3$ buffer (pH 8.0) similarly as above. The reacted gel was washed with three cycles of 0.1 M sodium acetate buffer, pH 4.0/1.0 M NaCl followed by 0.1 M sodium borate buffer, pH 8.0/1.0 M NaCl. Finally, the gel was washed with 0.1 M HCl to remove any somatostatin bound to the coupled immunoglobulin, rewashed with 0.1 M sodium phosphate buffer (pH 7.4), and stored in the sodium phosphate buffer containing 0.01% sodium azide. Previous studies in our laboratory had demonstrated that a small amount of IRS originating from sheep serum remained bound to the coupled antiserum throughout the preparation of the immunoaffinity gel and that acid elution removed the extraneous IRS (11). Prior to experimental use, a portion of the immunoaffinity gel was tested by elution with 0.1 M HCl/0.1% human serum albumin to ensure that the gel was free of pre-existing IRS.

A column (0.7 × 6.5 cm or 0.75 × 14 cm) of the immunoaffinity gel was equilibrated with 0.1 M sodium phosphate buffer, pH 7.4/0.1% human serum albumin. A pool of retinal extracts was neutralized with NaOH to pH 7.4 as determined by a pH meter, adjusted to 10 mM in sodium phosphate by addition of a 0.5 M sodium phosphate buffer (pH 7.4), centrifuged as described above, and applied to the immunoaffinity column at 4°C. The flow rate was 12.5 ml/hr, and 2.0-ml fractions were collected. After application of the retinal extracts, the column was rewashed with buffer. The reversibly bound IRS was eluted with 0.1 M HCl/0.1% human serum albumin. RIA was performed on each fraction.

Gel Filtration Chromatography. Sephadex G-25 fine was swollen overnight in 0.2 M acetic acid containing 0.1% human serum albumin. A portion of the IRS that eluted from the immunoaffinity column was freeze dried, resuspended in 0.6 ml of distilled H_2O, and applied (0.55 ml) to a 1.1 × 50 cm gel filtration column. Chromatography was performed at 4°C with an eluent of 0.2 M acetic acid/0.1% human serum albumin, a flow rate of 10 ml/hr, and a fraction size of 1.0 ml. A portion of each fraction was neutralized with NaOH and assayed for IRS. Synthetic somatostatin (200 ng) was chromatographed under similar conditions with a recovery of 51%. The incomplete recovery is probably due to binding of the peptide to the gel or to loss of immunological activity.

Experimental Models of Retinal Degeneration: Clues to Cellular Localization of IRS in Retina. IRS was measured in adult (250 g) PETH (pink-eyed, tan-hooded) rats and in albino (Osborne–Mendel) controls. PETH rats have inherited retinal dystrophy which begins to manifest itself in the third postnatal week (12, 13). The adult animals have few if any rods and gradually lose most of their cones as well. Thus, if IRS were confined to photoreceptor elements in the retina, PETH rat retinas should have little of this material.

In addition to studying PETH rats, we also examined the retinas of rats that had been subjected to intracranial bilateral

optic nerve transections. Rats (250 g) were placed in a Kopf stereotaxic device (5°, nose down) and a 3-mm knife with its blade in the coronal plane was centered over the midline and inserted into the brain 10-11 mm rostral to the interauricular line. The knife was lowered until it reached the base of the brain and pressed into the bone so that it cut both optic nerves. The animals were killed 1 year after the operation. After this time all of the ganglion cells had degenerated (14) and the optic nerves and tracts had completely atrophied. The retinas of the surgically prepared rats would be expected to have no IRS if it were localized exclusively in the ganglion cells.

RESULTS

Concentration of IRS in Rat Retina. The concentrations of IRS were 612 ± 43 (mean ± SEM) pg per retina or 0.621 ± 0.044 pg/μg of protein for a total of 16 retinas obtained from 10 rats killed by decapitation and 601 ± 22 pg per retina or 0.577 ± 0.022 pg/μg of protein for 19 retinas obtained from 10 rats killed by ether inhalation. The differences between the two groups were not statistically significant ($P > 0.1$, two-tailed t test).

Characterization of Retinal Somatostatin-Like Activity. Fig. 1 represents the immunoaffinity chromatography of a pool of retinal extracts containing a total of 37.9 ng of IRS. The concentration of IRS in the elution fractions corresponding to the application of the sample was below the RIA limit of detection (200 pg/ml). However, 52% of the applied IRS eluted when the column was washed with HCl. The immunoaffinity-purified retinal IRS produced a displacement curve of ^{125}I-labeled [Tyr^1]somatostatin from antiserum that was similar to those of synthetic somatostatin and rat hypothalamus extracted in HCl by the same method as used for retinas (Fig. 2). The displacement lines of crude retinal extracts were also parallel to that of synthetic somatostatin (data not shown).

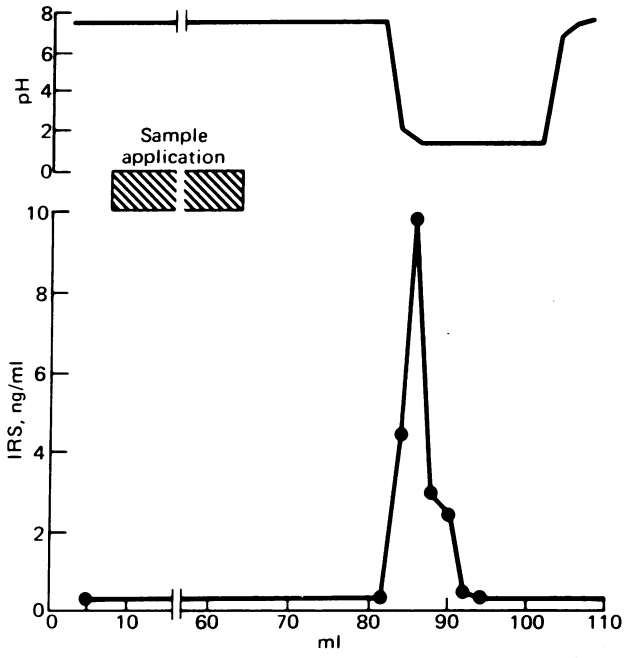

FIG. 1. Immunoaffinity chromatography of rat retinal IRS. The pH and concentration of IRS (ng/ml) determined on each 2-ml elution fraction are plotted against the cumulative elution volume. A total of 37.9 ng of IRS was applied in a volume of 55 ml to a 0.7 × 6.5 cm immunoaffinity column.

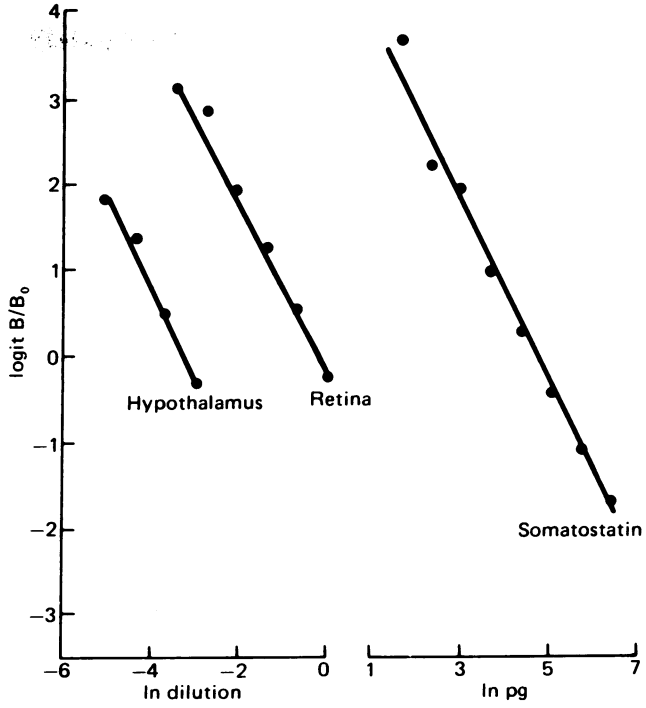

FIG. 2. Lines relating the displacement of ^{125}I-labeled [Tyr^1]-somatostatin from antiserum in response to dilutions of a rat hypothalamic extract, the immunoaffinity-purified retinal IRS prepared as shown in Fig. 1, and synthetic somatostatin under the conditions of the RIA. B/B_0 is the ratio of ^{125}I-labeled [Tyr^1]somatostatin bound in the presence of synthetic somatostatin or experimental sample to that bound in the absence of synthetic somatostatin. Each point is the mean of duplicate determinations. The lines are determined by a least-squares linear regression analysis: synthetic somatostatin ($y = -1.06x + 5.00$, $r = 0.9927$), immunoaffinity-purified retinal IRS ($y = -1.02x - 0.217$, $r = 0.9927$), hypothalamic extract ($y = -1.10x - 3.64$, $r = 0.9927$).

Fig. 3 indicates the dose–response curves in the somatostatin bioassay as determined by inhibition of rat growth hormone released into the incubation medium compared to varying quantities of somatostatin, crude retinal extract, and the immunoaffinity-purified IRS obtained from the chromatography described in Fig. 1. All three samples produced a dose-related inhibition of release of rat growth hormone. The ratios of somatostatin-like activity determined by bioassay to that determined by RIA were 0.84 and 0.79 for two separate immunoaffinity experiments and bioassays. The bioassay/RIA ratios of somatostatin-like activity assayed in two separate crude retinal extracts were 1.53 and 2.09.

Fig. 4 shows the chromatographic behavior on gel filtration of the IRS purified by immunoaffinity chromatography. A total of 45% of the IRS that eluted from the immunoaffinity column was recovered after lyophilization and gel filtration chromatography. Approximately 96% of the recovered IRS eluted in a peak at a volume similar to that of synthetic somatostatin.

Effect of Retinal Degenerations on IRS Concentration. As shown in Table 1, the concentration of retinal IRS, expressed as pg per μg of protein, is significantly increased in PETH rats with hereditary degeneration of photoreceptor cells and in rats studied 1 year after transection of the optic nerves. A decrease in the retinal protein concentration of the experimental groups accounts for the increase in retinal IRS concentration.

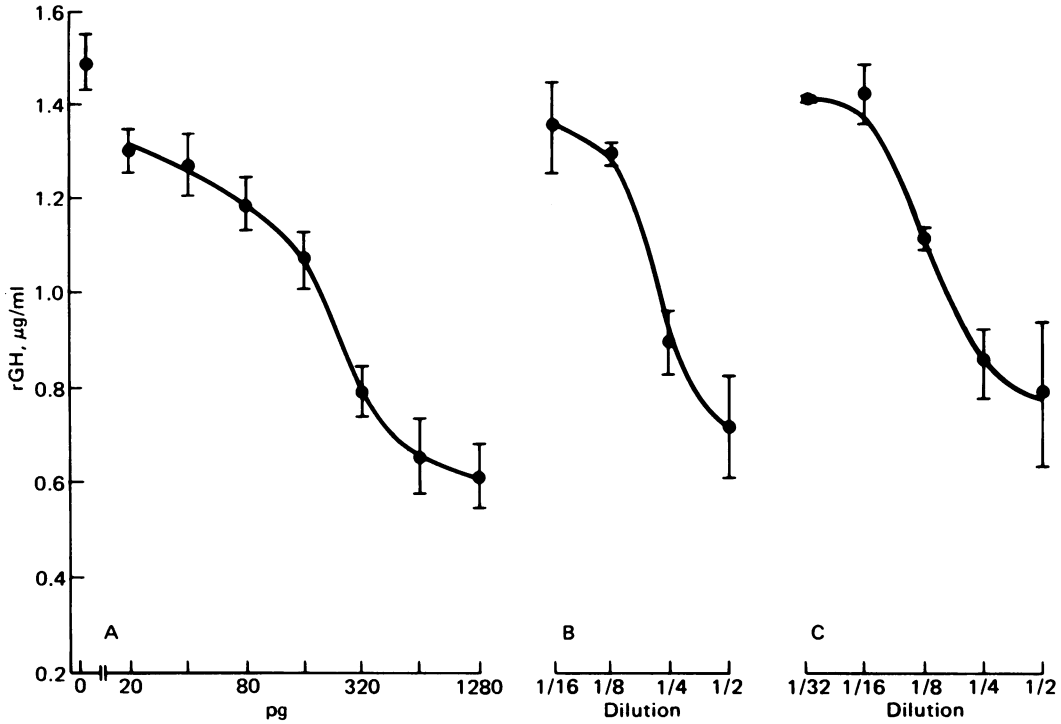

FIG. 3. Curves relating the concentration of rat growth hormone (rGH) released from cultured rat anterior pituitary cells into their culture medium and pg of synthetic somatostatin (*A*) or dilutions of a crude retinal extract (*B*) and immunoaffinity-purified IRS (*C*) added to the incubation medium. Each point represents the mean of rat growth hormone release in six incubation dishes for the synthetic somatostatin curve and the buffer control (0) (*A*) and three dishes for the retinal samples (*B* and *C*). The vertical lines represent the SEM.

DISCUSSION

The rat retina contains a material that exhibits immunological, biological, and chromatographic properties similar to those of somatostatin. The immunoaffinity chromatography experiments demonstrate that IRS binds reversibly to immobilized antisomatostatin immunoglobulin. The IRS that has thus been partially purified and concentrated retains the capacity to compete with ^{125}I-labeled [Tyr^1]somatostatin for antiserum binding in the RIA and to inhibit the release of growth hormone from rat anterior pituitary cells in a fashion comparable to that of crude retinal extracts and somatostatin. Because of the relatively low concentration of IRS in the retina and because attempts to perform gel filtration chromatography of IRS in crude brain extracts had not produced reliable results in our hands, we chose to use the IRS purified by immunoaffinity chromatography prior to gel filtration. The predominance of the single peak of retinal IRS, which elutes at a volume similar to that of somatostatin, contrasts to the more complex chromatographic patterns reported on gel filtration of IRS in extracts from other brain regions (15–18). Patel and Reichlin (15) studied extracts of rat hypothalamus and cerebral cortex under denaturing conditions and obtained one peak of IRS that coeluted with somatostatin and a smaller, though substantial, peak that appeared just after the void volume. Results from our laboratory derived from an immunoaffinity and gel filtration protocol similar to that used for the retina, but performed on acetic acid extracts of rat median eminence, anterior hypothalamus, amygdala, and cerebral cortex, indicated two peaks of IRS eluting at earlier volumes than synthetic somatostatin (16). Spiess and Vale (19), using gel filtration done under conditions

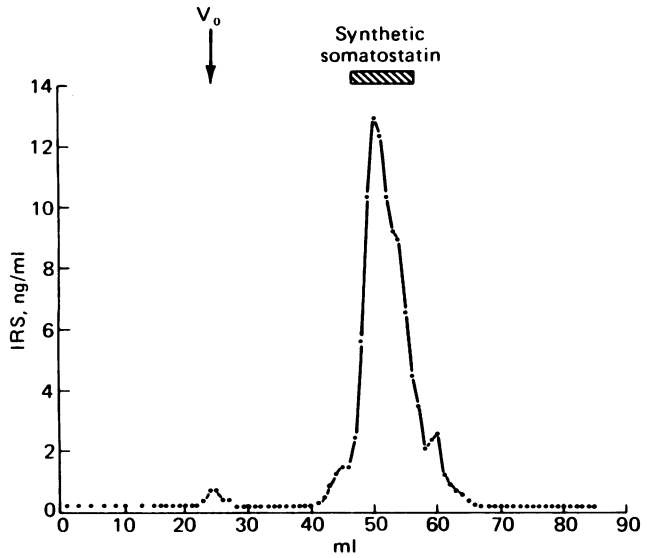

FIG. 4. Gel filtration chromatography of immunoaffinity-purified retinal IRS. The concentration of IRS per ml of elution fraction is compared to the cumulative elution volume. V_0 is the column void volume. The elution volume of synthetic somatostatin is indicated by a crosshatched bar.

Table 1. Effect of retinal degeneration on IRS concentration

Rats (n = 6)	Protein, μg/extract	IRS, pg/extract	IRS, pg/μg protein
Control	921 ± 20	879 ± 80	0.96 ± 0.09
Hereditary photoreceptor degeneration	454 ± 28*	725 ± 39	1.46 ± 0.16†
Optic nerve transection	474 ± 26*	1158 ± 168	2.40 ± 0.27*

Data are expressed as mean ± SEM.
* $P < 0.001$.
† $P < 0.01$, compared to control (two-tailed t test).

to dissociate noncovalent bonds, observed an early migrating species of IRS from rat hypothalamus and extrahypothalamic brain which accounted for 3–5% of total IRS. This proportion of an early migrating form is consistent with that which we observed for retinal IRS. Each of the gel filtration studies of rat brain IRS used antisera that recognize the central portion of the somatostatin molecule. At present it is difficult to resolve the differences observed between separate laboratories and between different brain regions. However, it is common to all groups that they have noted forms of IRS extracted from all brain regions studied which elute earlier on gel filtration than synthetic somatostatin, although these forms vary with respect to the proportion of total IRS that they constitute. Whether these early eluting species represent biosynthetic precursors of somatostatin remains to be established.

The concentration of IRS in the retina is less than that reported for the majority of rat brain regions, including the hypothalamus, amygdala, cerebral cortex, brainstem, and spinal cord (7, 15, 20–22). Rat brain areas that contain levels of IRS comparable to that found in the retina are: pineal, 0.14–0.5 ng/mg of protein (15, 20, 22) or 250 pg/gland (7); cerebellum, 0.43 and 0.40 ng/mg of protein (15, 22); substantia nigra, 0.9 and 0.3 ng/mg of protein (20, 22); and interpeduncular nucleus, 1.0 and 0.4 ng/mg of protein (20, 22).

The distribution of IRS in the rat nervous system is complemented by the results of pituitary cell bioassay studies of Vale *et al.* (23). This group has studied extrahypothalamic rat brain by bioassay and three different RIAs and have observed ratios of bioassayable somatostatin to IRS of 1.26, 2.16, and 1.86§ (24). It is consistent with these observations that the bioassay/RIA ratios determined on crude retinal extracts are also above unity. This, taken together with the fact that the bioassay/RIA ratio of somatostatin purified by immunoaffinity chromatography was less than unity, suggests the presence of material in the crude retinal extract that inhibits the release of growth hormone but does not react with our antisomatostatin serum. Interestingly, both reports of the initial purification of somatostatin from sheep and porcine hypothalami contain evidence of preparative fractions with growth hormone release-inhibiting activity that are distinct from the fractions from which somatostatin was isolated (25, 26).

Our studies do not determine in which cellular elements of the retina somatostatin-like material is localized. The fact that IRS content is preserved in experimental models of photoreceptor and ganglion cell degeneration argues against localization in these cells. The optic nerve transection data also indicate that the retinal content of IRS is independent of central connections. This observation is consistent with either local biosynthesis or uptake of IRS from blood or vitreous humor. Although the data presented are considerably short of the unequivocal identification that can be achieved only by the isolation from the retina of an amino acid sequence identical to that of somatostatin, we are encouraged that the retina may prove a useful model tissue for the study of the relationship between somatostatin and neuronal function.

Note Added in Proof. After submission of this paper, a report of IRS in extracts of a tissue piece consisting of rat sclera, choroid, and retina appeared (27).

§ Calculated from data in table 1 of ref. 24.

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