Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 11485–11489, December 1991
Physiology

Evidence that somatostatin is localized and synthesized in lymphoid organs

(periventricular hypothalamic nucleus/spleen/thymus/bursa of Fabricius/B and T lymphocytes)

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Contributed by S. M. McCann, September 23, 1991

ABSTRACT **Because several peptides originally found in the pituitary as within the central nervous system have been localized in lymphoid tissues and because somatostatin (somatotropin-release-inhibiting hormone, SRIH) can act on cells of the immune system, we searched for this peptide in lymphoid organs. We demonstrated that SRIH mRNA exists in lymphoid tissue, albeit in smaller levels than in the periventricular region of the hypothalamus, the brain region that contains the highest level of this mRNA. SRIH mRNA was found in the spleen and thymus of male rats and in the spleen, thymus, and bursa of Fabricius of the chicken. Its localization in the bursa indicates that the peptide must be present in B lymphocytes since this is the site of origin of B lymphocytes in birds. The SRIH concentration in these lymphoid organs as determined by radioimmunoassay was greater in the thymus than in the spleen of the rat. These concentrations were 50 times less than those found in the periventricular region of the hypothalamus, the site of the perikarya of SRIH-containing neurons. In the chicken, as in the rat, the concentration of SRIH was greater in the thymus than in the spleen; it was present in the bursa of Fabricius, also in higher concentration than in the spleen. Fluorescence immunocytochemistry revealed the presence of SRIH-positive cells in clusters inside the white pulp and more dispersed within the red pulp of the spleen of both the rat and the chicken. The thymus from these species also contained SRIH-positive cells within the medulla and around the corticomedullary junction. In the chicken, there were large clusters of SRIH-positive cells in the medullary portion of each nodule of the bursa of Fabricius. Preabsorption of the primary antiserum or replacing this antiserum with normal rabbit serum verified the specificity of staining. Sequential immunostaining of the same sections from rat spleen using first SRIH antibody and subsequently a monoclonal antibody against a rat B-cell surface antigen revealed the presence of SRIH immunoreactivity in some, but not all, B cells. Other cell types in spleen not yet identified also stained positively with the SRIH antibody but were not reactive to monoclonal antibodies to rat Thy-1.1, a marker for all the thymic T lymphocytes. The possibility that SRIH is present in other populations of cells in the spleen cannot be ruled out. Sequential immunostaining of the same sections of rat thymus revealed the presence of SRIH immunoreactivity in a small population of T lymphocytes in the medulla, as revealed by the Thy-1.1 marker. The SRIH-positive cells were nonimmunoreactive when exposed to the B-cell marker; however, the possibility that SRIH is present in other cells was not investigated. Thus, our results indicate that SRIH is synthesized and stored in cells of the immune system. SRIH may be secreted from these cells to exert paracrine actions that alter the function of immune cells in spleen and thymus.**

Somatostatin (somatotropin-release-inhibiting hormone, SRIH), originally described and isolated from the hypothalamus (1, 2) by its ability to inhibit growth hormone release occurs in a variety of endocrine and nonendocrine tissues, including other parts of the brain, pancreas, stomach, and duodenum (3, 4). Such a wide distribution of SRIH implies the hormone has a larger functional significance that extends beyond the regulation of growth hormone secretion. There is increasing evidence that SRIH influences numerous bodily functions including the immune response. Specific receptors for SRIH have been found on lymphocytes and monocytes (4, 5); it exerts both stimulatory and inhibitory effects on lymphocyte proliferation (5–8), inhibits the release of colony-stimulating factor from activated lymphocytes (9), and suppresses immunoglobulin synthesis (5). In contrast to these inhibitory actions, SRIH enhanced leukocyte-migration-inhibiting factor formation in activated lymphocytes (5). This study was conducted to investigate the presence of SRIH and its mRNA in the spleen and thymus of the rat and chicken and in the bursa of Fabricius of the chicken, the organ that exclusively produces B lymphocytes in birds.

MATERIALS AND METHODS

Experimental Animals. Thirty-day-old male Sprague–Dawley-derived rats (Holtzmann, Madison, WI) and chickens, which were 7-week-old Leghorn poults, were used as tissue donors. The rats were housed in group cages (10 rats per cage) under controlled conditions of light (hours lights on 0500–1900) and temperature [24 ± 1°C (mean ± range)]. Rat Chow and water were provided ad libitum. The chickens were normal healthy birds from the Texas A & M University poultry farm. The animals were killed by decapitation, and the tissue samples were quickly collected, frozen in liquid nitrogen, and stored at −70°C until analyzed.

Plasmid Preparation. A cDNA probe for rat prepro-SRIH in expression vector pSP65 (10) was generously provided by R. H. Goodman (University of Oregon School of Medicine, Portland, OR). *Escherichia coli* Y1088 were transformed with this vector using the calcium chloride procedure (11) and the culture was expanded in LB broth containing ampicillin (50 μg/ml). The plasmid was isolated by the alkaline lysis method (12) and purified with polyethylene glycol (12). The presence of the SRIH cDNA probe was initially confirmed by digestion with the restriction enzymes *Xba* I and *Bam*HI followed by separation on a 1% agarose gel and staining with ethidium bromide. An insert of ≈500 base pairs was detected, which is consistent with the size of the rat prepro-SRIH cDNA insert (13).

SRIH cRNA Probe Synthesis and Labeling. The transcription mixture (10 μl) contained 0.25 μg of *Sal* I-digested SP65 cDNA, 7 units of SP6 RNA polymerase, 10 units of human placenta RNase inhibitor, 40 mM Tris·HCl (pH 7.9), 1 mM dithiothreitol, 0.5 mM ATP, 0.5 mM GTP, and 0.5 mM UTP;

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Abbreviations: SRIH, somatotropin-release-inhibiting hormone; PVN, periventricular nucleus.

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25 μM [α-^{32}P]CTP (800 Ci/mmol; 1 Ci = 37 GBq) was the substrate in the synthesis of the RNA probe that was used in quantitation of SRIH mRNA. After incubation at 37°C for 1 h, the transcription mixture was digested with 1 unit of DNase I at 37°C for 10 min, extracted with phenolchloroform, 1:1 (vol/vol), and purified by two precipitations with 2 vol of ethanol per precipitation in the presence of yeast tRNA (0.7 mg/ml). The RNA probe was washed in ice-cold 70% ethanol (pelleted by centrifugation) and dissolved in Tris/EDTA (TE = 10 mM Tris·HCl, pH 8.0/1 mM EDTA).

Transcript Specificity. Control experiments were performed to demonstrate the specificity of the binding of the probe for these assays. All controls performed [i.e., S1 nuclease digestion and hybridization with tRNA and with sense RNA for tyrosine hydroxylase, kindly provided by J. C. Porter (University of Texas Southwestern Medical Center, Dallas)] resulted in the absence of the nuclease-resistant fragment.

Quantification of SRIH mRNA. Total mRNA was extracted using the guanidinium/hot phenol method of Feramisco *et al.* (14). Spleen and thymus from rat and chicken and bursa of Fabricius (<20 mg) were homogenized in 0.35 ml of extraction buffer (4.5 M guanidinium thiocyanate/25 mM sodium citrate, pH 7.0/10 mM EDTA/1% Sarkosyl/1% 2-mercaptoethanol) and extracted with 0.85 ml of a mixture of phenol, 0.1 M sodium acetate (pH 5.2), and chloroform, 35:15:35 (vol/vol). Total RNA (10 μg) was hybridized with 50,000 cpm of ^{32}P-labeled SRIH RNA probe per tube and subjected to an S1 nuclease protection assay (15) as adapted by Kedzierski and Porter (16). RNA probe protected by duplex formation with SRIH mRNA was analyzed by polyacrylamide gel (8% gel/8 M urea) electrophoresis, followed by autoradiography and densitometry.

RIA of SRIH. The quantity of SRIH present in the tissues was determined by RIA as described (17) with minor modifications. The highly specific antiserum (R-11C) was generously provided by Louis De Palatis (Dow Chemical). The characterization of this antiserum has been described (18). In brief, the antiserum did not crossreact at a 100-fold molar excess with luteinizing-hormone-releasing factor, vasoactive intestinal peptide, thyrotropin-releasing hormone, α-melanocyte-stimulating hormone, cholecystokinin (1–8), substance P, and β-endorphin. R-11C crossreacts fully with synthetic SRIH (SRIH-14) and 40% with SRIH-28. All results were expressed as pg of SRIH released per mg of protein.

Protein Measurement. Protein was measured by the procedure of Lowry *et al.* (19) using bovine serum albumin as the standard.

Morphological Analyses. The immunofluorescence protocol used is identical to that described (20, 21). The following antisera were used: R-11C, as described above, or JH202 (kindly supplied by S. Kentroti, University of Colorado, Denver, CO). Both antisera yielded similar results and staining was evident at dilutions from 1:100 to 1:1600. The addition of 500 μg of SRIH-14 and 500 μg of SRIH-28 to 1 ml of a 1:100 dilution of antiserum resulted in a 85–90% reduction of immunostaining. Preabsorption of a 1:1000 dilution of antiserum eliminated all immunostaining when compared with nonpreabsorbed antisera at this same dilution. Additionally, specificity of immunostaining was further verified by using normal rabbit serum in place of the primary antisera.

To determine whether SRIH is present in B or T lymphocytes, antibodies to rat lymphocyte cell surface antigens were used. A monoclonal antibody (Thy-1.1) that recognizes the Thy-1 antigen expressed in all the thymic lymphocytes was purchased from Amgen Biologicals. Another monoclonal antibody (MARK-I, 1:1000 dilution), which recognizes the surface immunoglobulin present in rat B cells, was generously provided by J. W. Uhr (Department of Microbiology, University of Texas Southwestern Medical Center).

Some sections were counterstained with hematoxylin/eosin or alcian blue/periodic acid-Schiff (AB/PAS) for the histological analysis of red and white splenic pulp or for the presence of mast cells in the thymic corticomedullary junction, respectively.

RESULTS

Validation of the mRNA Method. To evaluate the efficiency of the quantification of SRIH mRNA: several amounts of total cytoplasmic RNA (5, 10, or 20 μg) were hybridized with the ^{32}P-labeled SRIH RNA probe. The protected RNA duplex was analyzed by polyacrylamide gel electrophoresis and quantified by autoradiography and densitometry. The resulting RNA concentration/absorbance curve demonstrated a linear relationship between the amount of RNA added and the intensity of the reaction (data not shown).

Localization of SRIH mRNA in Hypothalamus and Organs of the Immune System. Cytoplasmic RNA was isolated from young male rats from tissue from the periventricular nucleus (PVN) region of the hypothalamus and from the spleen and probed for the presence of specific SRIH mRNA with an RNA probe for rat SRIH. Fig. 1 demonstrates the presence of SRIH mRNA in the PVN and to a lesser extent in spleen. From the autoradiogram in Fig. 2, it is apparent that the PVN and the spleen hybridize to a species of mRNA that is ≈600 bases and was similar to that previously reported (22, 23).

Having identified the SRIH mRNA in the spleen, we examined also the thymus of the rat and the spleen, thymus, and bursa of Fabricius of the chicken. The latter organ was selected since it is the exclusive site for formation of B lymphocytes in birds. The SRIH mRNA levels from rat and chicken thymus were detectable (Fig. 2) and were higher than those found in the spleen of either species. In the chicken, the bursa of Fabricius had even greater levels of SRIH mRNA than those found in spleen or thymus. The levels of SRIH mRNA in all of these organs were significantly less than those found in the PVN of the hypothalamus, which contains the highest level of mRNA for SRIH observed in any organ.

SRIH Concentrations in Organs of the Immune System. Since SRIH mRNA was present in these organs, it was anticipated that SRIH could also be detected by RIA. Indeed, low and approximately equivalent concentrations of SRIH were found in both rat and chicken spleen (Table 1); there were greater levels in the thymus of both species. The bursa of Fabricius from the chicken had much higher concentrations than those present in the spleen of the chicken and levels somewhat less than those found in chicken thymus.

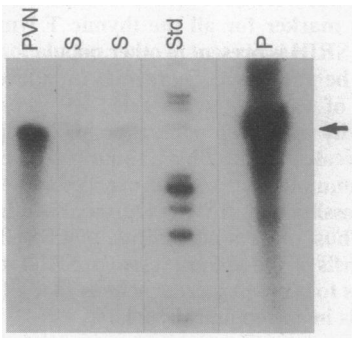

FIG. 1. Representative autoradiogram of SRIH mRNA in spleen (S) and PVN. Molecular weight standards (Std, φX174re DNA/*Hae* III fragments) and probe (P) are also shown. The arrow indicates a standard band size of 603 base pairs. Total RNA (10 μg) was hybridized and analyzed as above. Values are listed as densitometric units in 10 μg of total cytoplasmic RNA isolated from the tissues previously described. They were as follows: PVN, 132; S, 32 and 38.

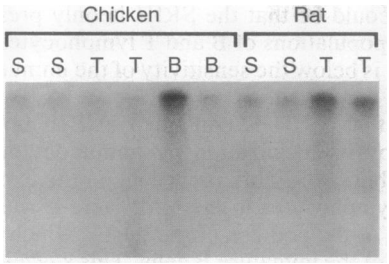

FIG. 2. S1 nuclease protection assay of SRIH mRNA in 10 μg of total RNA in spleen (S) and thymus (T) from rat and chicken, and in bursa of Fabricius (B) of the chicken.

The concentrations of SRIH in all these organs were significantly less than those found in PVN (1362 ± 18 pg of SRIH per mg of protein).

Localization of SRIH in Lymphoid Organs by Immunocytochemistry. Immunocytochemical staining of 10-μm frozen sections mounted on coverslips revealed that SRIH-positive cells were present in large clumps within both rat (Fig. 3*A*) and chicken (data not shown) spleen. Some sections were counterstained with hematoxylin/eosin and examination of these sections verified that some of these clumps of SRIH cells were clearly within the splenic white pulp. Other positive cells, which were outside these clumps and were more dispersed (Fig. 3*C*), were localized to the red pulp.

In some experiments, after immunofluorescence and photography of the SRIH-positive cells in the rat, tissues were washed in phosphate buffer then subjected to a counterstain using specific monoclonal antibodies to either B or T lymphocytes. This reaction employed the Vectastain ABC procedure utilizing diaminobenzadine as the chromogen. Fig. 3 *B* and *D* shows the same fields as shown in Fig. 3 *A* and *C*, respectively. As can be seen with this low magnification, the large clump of SRIH-positive cells (Fig. 3*A*) in spleen were found to be predominantly B lymphocytes as revealed by using the antiserum (MARK-I) to the B-cell surface antigen (Fig. 3*B*). Importantly, all B lymphocytes were not positive for SRIH and, conversely, some SRIH-positive cells may be yet another cell type. This becomes more apparent by examining the more dispersed cells depicted in Fig. 3 *C* and *D*. Additionally, counterstaining SRIH-positive cells in spleen using antiserum to the surface antigen Thy-1.1 revealed no positive immunostaining in rat T cells (data not shown). These results do not rule out the possibility that other cells may contain SRIH within the spleen.

Similarly, the thymus from both species exhibited intense SRIH-positive cells in small clusters within the medulla, with less intensely stained cells around the corticomedullary junction. Fig. 4 depicts a representative section of the rat thymus. Some sections of the rat thymus were then counterstained with the MARK-I, B-cell surface marker as described above, and results indicate that the SRIH-positive cells were not B lymphocytes. Other sections were counterstained with anti-Thy-1.1, the T-cell marker, and those results demonstrate the presence of SRIH immunoreactivity in a small population of T lymphocytes at the corticomedullary junction (Fig. 5).

Table 1. Concentrations of SRIH in the immune system organs

Species	Tissue	*n*	SRIH, pg/mg of protein
Rat	Spleen	4	34.44 ± 6.6
	Thymus	5	78.10 ± 7.9*
Chicken	Spleen	4	42.4 ± 4.7
	Thymus	3	197.8 ± 28.5†
	Bursa of Fabricius	3	129.6 ± 2.5†

Data are mean ± SEM. *, $P < 0.005$ vs. rat spleen; †, $P < 0.001$ vs. chicken spleen.

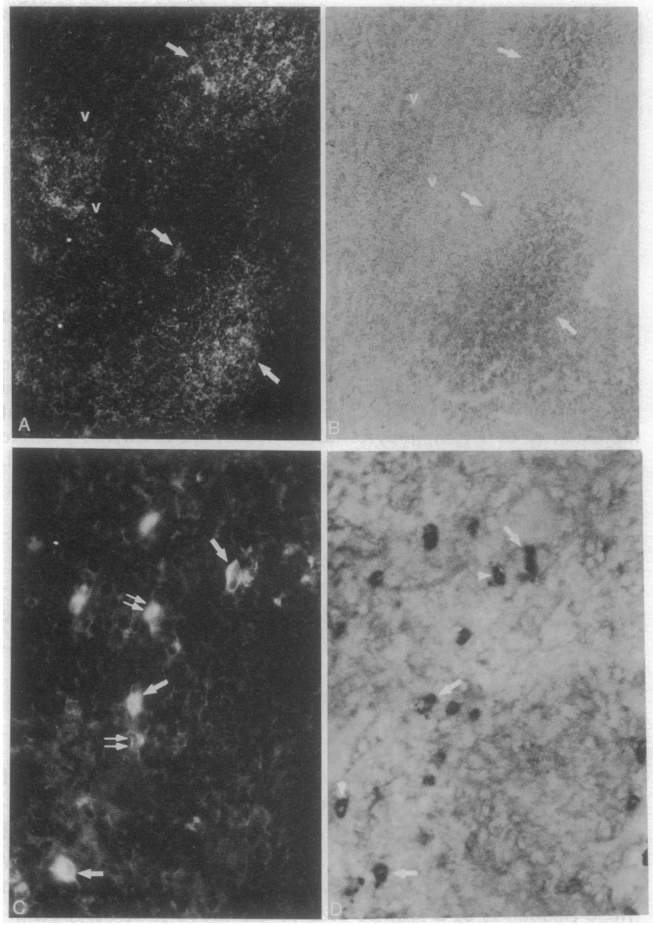

FIG. 3. Presence of SRIH-immunoreactive B lymphocytes in the rat spleen. (*A*) Distinct clumps of splenic cells (arrows) positive for SRIH by immunofluorescent staining. v, Vessel. (×70.) (*B*) Same field depicted in *A* demonstrating that the clumps of SRIH-positive cells are also shown to stain positive with monoclonal antibodies to a rat B-lymphocyte surface antigen. (×70.) (*C*) Several dispersed SRIH-positive (single arrows) cells in the red pulp. (×175.) (*D*) Same field depicted in *C* demonstrating that the same SRIH-positive cells are B lymphocytes (arrows). (×175.) By comparing *C* and *D*, it can also be seen that some of the B cells shown in *D* (arrowheads) are not SRIH-positive (see *C*), and likewise, some SRIH cells shown in *C* (double arrows) are not B lymphocytes (see *D*).

Again, these results do not rule out the possibility that other cells in the thymus, possibly macrophages, may contain SRIH. In a separate experiment the possibility that the positive SRIH cells at the corticomedullary junction were

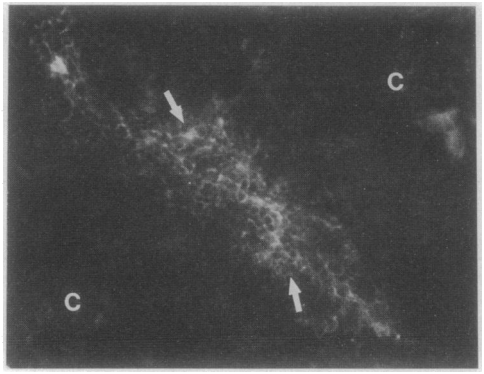

FIG. 4. Immunoreactive SRIH cells in the rat thymus. Note that the majority of immunostaining was present in the medullary (arrows) portion of this thymic lobule. c, Cortex. (×175.)

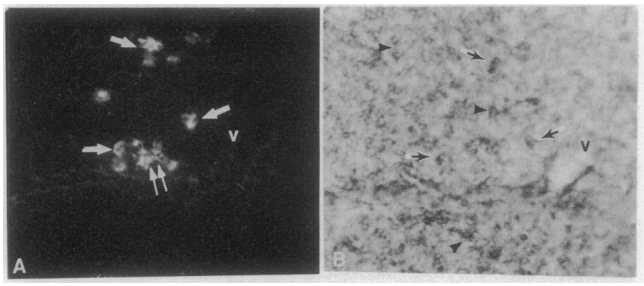

FIG. 5. Presence of SRIH-positive T lymphocytes in the rat thymus. (*A*) A few SRIH-positive cells (arrows) at the corticomedullary junction. (*B*) Same field depicted in *A* demonstrating that some of these SRIH-positive cells are T lymphocytes (arrows), as revealed by a monoclonal antibody to Thy-1.1. These photos also show that the majority of T lymphocytes (arrowheads) are not SRIH-positive and that some SRIH cells (double arrows) are not T cells. v, Vessel. (×135.)

mast cells was ruled out by the alcian blue/periodic acid-Schiff counterstain procedure.

The chicken bursa of Fabricius also contained SRIH-positive cells. In this organ, the immunoreactive cells were largely confined to the medullary portion of each nodule (Fig. 6).

DISCUSSION

In the present study we have characterized the presence of SRIH-specific mRNA in spleen, thymus, and bursa of Fabricius by an S1 nuclease protection assay using a SRIH complementary RNA probe. These tissues contained only one species of SRIH mRNA. The length of the message in these tissues is of a similar size to that seen in the hypothalamus (22) and PC-12 cells (23). The SRIH concentrations in these tissues analyzed by RIA showed a similar pattern as that seen with SRIH mRNA. These findings were corroborated by our immunocytochemical studies showing SRIH-positive cells in thymus, spleen, and bursa of Fabricius. Finally, double staining revealed that some but not all B lymphocytes in rat spleen contained SRIH and that this peptide was also contained in other splenic cells that have not been identified. Additionally, SRIH was not observed in a population of T lymphocytes that express the Thy-1.1 surface antigen. In the thymus, SRIH was not contained in B lymphocytes but was found instead to be present in T lymphocytes. The failure to observe SRIH in some B and T cells does not mean that these cells do not contain the peptide. It could be that the SRIH is only present in some specific subpopulations of B and T lymphocytes and/or that the quantity is below the sensitivity of the immunocytochemical method employed.

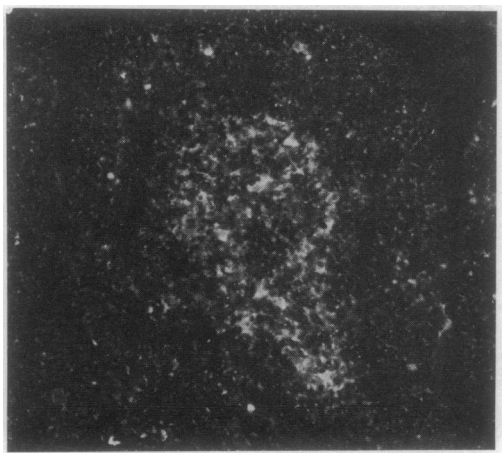

FIG. 6. SRIH-positive cells in a representative nodule of the bursa of Fabricius of the chicken. Note the more intense immunoreactivity in the medullary portion of this nodule. (×138.)

The fact that SRIH is contained in B lymphocytes was confirmed by its localization by immunocytochemistry to cells of the bursa of Fabricius, which is the exclusive organ forming B lymphocytes in the bird. Here it was detected in the medulla of the nodules of the bursa, which is the site of localization of the immature B cells. This suggests that SRIH is present in greater abundance in immature rather than adult B cells.

Our results provide strong evidence that SRIH is synthesized in and is present in lymphocytes in the organs of the immune system. These observations strongly suggest that SRIH may play a role in regulating the activity of these cells. It is probably released from these cells to exert paracrine actions on adjacent immune cells in lymphoid organs. Indeed, a number of such actions have been characterized. SRIH can either inhibit or stimulate lymphocyte proliferation (5–8), inhibit the release of colony-stimulating factor from activated lymphocytes (9), and suppress immunoglobulin synthesis (5), whereas it enhances leukocyte migration-inhibiting factor formation in activated lymphocytes (7). These actions are probably mediated by combination of SRIH with its specific receptors, which have been localized on the surface of lymphocytes and monocytes (4, 5).

The evidence is mounting that many peptides produced in the brain and gastrointestinal tract are contained within immune cells. Examples include adrenocorticotropin hormone (24), β-endorphin (25), vasoactive intestinal polypeptide (26, 27), thyrotropin (28), chorionic gonadotropin (29), follicle-stimulating hormone (30), luteinizing hormone (30), growth hormone (31), and finally growth-hormone-releasing hormone (32). In this latter connection, it is tempting to speculate that growth-hormone-releasing hormone and SRIH might have antagonistic paracrine actions on immune cells just as they affect the release of growth hormone from the pituitary gland in an opposing manner. Thus, the immune system and the neuroendocrine system can produce neuropeptides, as well as interleukins and other cytokines, and also shares receptors for these neuroendocrine and immune mediators.

We thank Drs. Vijayakumar Boggaram, Carole R. Mendelson, and J. C. Porter for their helpful advice and suggestions for establishing the methodology and Dr. R. H. Goodman for providing us with recombinant SRIH cDNA. We would also like to thank Trae Lara and Jeff Minks for their excellent technical assistance and Judy Scott for typing the manuscript. This work was supported by National Institutes of Health Grants DK10073 (S.M.M.), DK40994 (S.M-.McC.), R29-NS26821 (M.C.A.), and AA00104 (W.L.D.).

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