Expression of Preprotachykinin-A and Neuropeptide-Y Messenger RNA in the Thymus

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The preprotachykinin-A gene, the common gene of mRNAs encoding both substance-P (SP) and neurokinin-A (NKA), was shown to be expressed in Sprague-Dawley rat thymus by detection of specific mRNA in gel-blot analyses. *In situ* hybridization revealed dispersed PPT-A-labeled cells in sections from rat thymus, with a concentration of grains over a subpopulation of cells in the thymic medulla. Also, neuropeptide-Y mRNA-expressing cells were found in the rat thymus, primarily in the thymic medulla. Rat thymic extracts contained SP-like immunoreactivity (SP-LI), and the major part of the immunoreactivity coeluted with authentic SP and SP sulfoxide standards. SP-LI was also detected in human thymus, which contained between 0.09–0.88 ng SP-LI/g wet wt. Evidence for translation of preprotachykinin-A mRNA in the rat thymus was obtained from the demonstration of NKA-LI in thymic cells with an epithelial-like cell morphology. Combined with previous observations on the immunoregulatory roles of tachykinin peptides and the existence of specific receptors on immunocompetent cells, the demonstration of intrathymic synthesis of NKA suggests a role for NKA-LI peptides in T-cell differentiation in the thymus. (Molecular Endocrinology 4: 1211–1218, 1990)

INTRODUCTION

T-Cell differentiation occurs primarily in the thymus (1, 2), and a substantial part of this complex program is regulated through direct cell to cell contacts as well as by paracrine factors locally synthesized within the thymic environment (3–5). A number of intrathymic paracrine factors have been isolated and characterized, including thymic hormones (6–9), colony-stimulating factors, and cytokines (10–12), which are synthesized mainly by epithelial cells. In addition, antigenic markers associated with different neuroendocrine cell types are found within the thymus, such as the surface complex ganglioside A2B5 (13), the secretory granule-associated protein chromogranin-A (14), neuron-specific enolase (15), and the surface glycoproteins HISL-5, -9, -14 (16), and -ID4 (17). In addition, recent reports from a number of laboratories have described the prescence of neuropeptides in the thymus, although some species differences may exist in the neuropeptide repertoire detected in the organ. Neurotensin-like immunoreativity (LI) and somatostatin-LI have been identified and biochemically characterized in the chicken thymus (18). Oxytocin (OT)-LI and neurophysin-LI have also been described in the human thymus (19), while vasopressin (VP)-LI has been characterized in murine and rat thymus (20, 21). Evidence for an intrathymic expression of these peptides has been obtained by positive hybridization in dot blot assays of thymic mRNA using OT and VP cDNA probes (22). OT-, VP-, and neurophysin-LI were identified by immunocytochemistry in thymic subcapsular cortex and medulla and in a specific subpopulation derived from the outer cortex, the thymic nurse cells (9, 15, 22). Met-Enkephalin-LI has also been detected in medullary and cortical surface epithelium (23). Recently, somatostatin mRNA has been demonstrated in the rat thymus, thus providing evidence for an intrathymic synthesis of this neuropeptide (24). A local im-

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munoregulatory function have been suggested for some of these neuropeptides, in parallel with the observation of specific receptors within the thymus or on immunocompetent cells (25–28).

The peptides substance-P (SP) and neurokinin-A (NKA) belong to a family of related peptides, the tachykinins, that are widely distributed in both the central and peripheral nervous system where they function as neurotransmitters/neuromodulators (29). Recently, Geppetti *et al.* (30, 31) detected SP-LI and NKA-LI in the rat thymus that was shown to migrate largely with synthetic SP and NKA, respectively, on reverse phase HPLC. Capsaicin pretreatment of rats was shown to decrease, but not abolish, SP-LI and NKA-LI in the thymus, suggesting that the detected thymic SP-LI and NKA-LI were derived mainly from known sensory innervation of the organ.

In this study we demonstrate expression of two neuropeptide mRNAs in the rat thymus, preprotachykinin-A (PPT-A) mRNA, encoding both SP and NKA (32), and NPY mRNA. RIAs using antisera specific for SP also showed the presence of authentic SP in both rat and human thymuses. In addition, immunohistochemical staining revealed the presence of NKA-LI in rat thymus. Hence, PPT-A-related peptides may be locally synthesized in the primary lymphoid organ, and since specific SP receptors and actions have been described on a subpopulation of T-cells (33, 34), we suggest that intrathymic tachykinin peptides may play a role in T-cell differentiation.

RESULTS

Tissue distribution of rat PPT-A mRNA

To study the tissue distribution of PPT-A mRNA, polyadenylated RNA was prepared from several adult Sprague-Dawley rat tissues, fractionated on denaturing agarose gels, and blotted to nitrocellulose filters, followed by hybridization to a ^{32}P-labeled rat PPT-A cDNA probe common to both SP and NKA. As expected (32), high levels of a 1.3-kilobase (kb) long PPT mRNA was found in the brain. In addition, a same size PPT mRNA was found in the rat thymus (Fig. 1). The level of PPT mRNA in the thymus, measured by densitometer scanning of autoradiograms from three independent experiments, was approximately 10 times lower than the level of PPT mRNA in rat brain. No other rat tissue analyzed, including the spleen, contained detectable levels of PPT mRNA. Furthermore, no PPT transcript was detected in mRNA prepared from isolated rat spleen cells (data not shown).

Localization of PPT-A mRNA in the Rat Thymus by *in situ* Hybridization

The rat PPT-A cDNA probe showed dispersed labeling over sections from the rat thymus, with the highest labeling occurring over the thymic medulla (Fig. 2, a and b). Analysis of the sections at higher magnification by emulsion autoradiography revealed the highest concentration of grains overlying a subpopulation of dispersed cells in the medulla (Fig. 3a). No, or very few, labeled cells were seen in the thymic cortex or the subcapsular region. No specific labeling was seen using a rat brain-specific cDNA clone also expressed in mitogen-stimulated spleen cells, but not in the thymus, included as a negative hybridization control (clone NI-2, Ericsson, A., G. Barbany, and H. Persson, manuscript submitted for publication; Figs. 2, e and f, and 3c). Moreover, no labeling over sections from rat thymus was seen using vector sequences (pUC 9 DNA) as a negative hybridization control (data not shown).

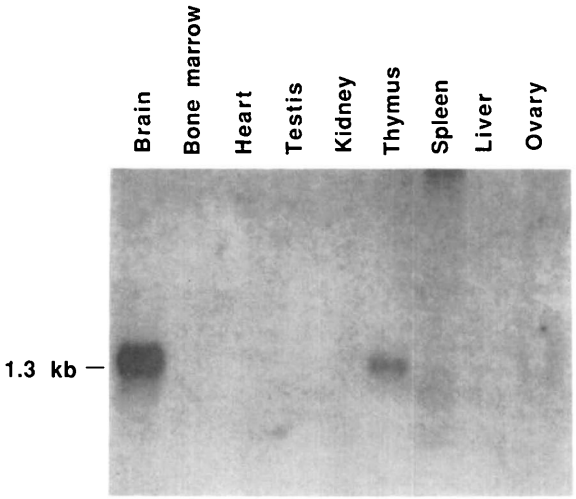

Fig. 1. Expression of PPT mRNA in Various Rat Tissues
Polyadenylated RNA (20 μg/slot) prepared from the indicated tissues of adult Sprague-Dawley rats was electrophoresed in a 1% agarose gel containing 0.7% formaldehyde. The RNA was then transferred to a nitrocellulose filter and hybridized to a nick-translated 300-bp *Bgl*II-*Bst*EII fragment from a rat PPT cDNA clone. The filter was washed at high stringency, followed by exposure to x-ray film.

Localization of NPY mRNA-Expressing Cells in Rat Thymus

The rat NPY gene has previously been shown to be expressed at a low level in the rat thymus (35). A DNA probe specific for rat NPY mRNA (35) was therefore used in an attempt to localize neuropeptide-Y (NPY) mRNA expressing cells in the rat thymus. This probe revealed specific labeling over sections from rat thymus (Fig. 2, c and d). As in the case of PPT, NPY mRNA-expressing cells in the thymus were found in the medulla, where a small subpopulation of cells was labeled (Fig. 3b). Both the PPT and NPY probes showed the expected neuronal labeling pattern on sections from rat brain hybridized in parallel with the thymus sections (data not shown).

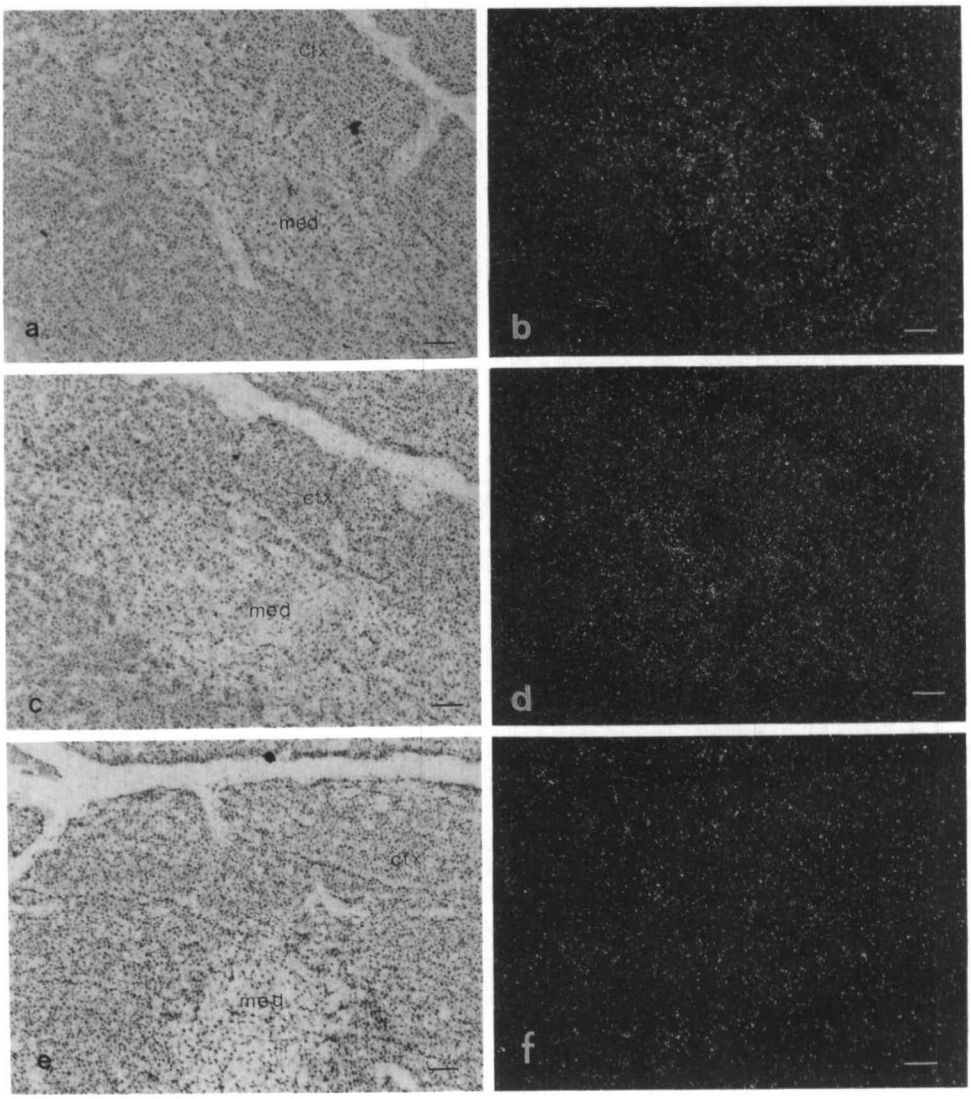

Fig. 2. *In Situ* Hybridizations to Sections from Rat Thymus

Cryostat sections from adult Sprague-Dawley rat thymus were hybridized to a 300-bp *Bgl*II-*Bst*EII fragment from a rat PPT cDNA clone (a and b), a 287-bp *Xho*I-*Ava*I fragment containing the second exon of the rat NPY gene (c and d), and a 500-bp *Eco*RI fragment from a rat cDNA clone, designated NI-2, not expressed in the thymus (e and f). All probes were labeled to the same specific activity with [α-^{35}S]dATP by nick translation. a, c, and e are brightfield micrographs and b, d, and f are darkfield micrographs obtained after emulsion autoradiography of the sections. ctx and med denote thymic cortex and medulla, respectively. Note the concentration of labeling over the thymic medulla in b and d. *Scale bars* are 125 μm.

RIA of SP-LI in Rat and Human Thymus Extracts

SP-LI was detected with either of two polyclonal SP antisera in a pool of rat thymuses as well as in each of five different human thymuses. The dilution curves of the different samples were strictly parallel to the standard curves of the RIAs. Figure 4 shows the results obtained with polyclonal SP_A antiserum. Concentrations of SP-LI were in the same range using either of the two polyclonal antisera in the RIAs [0.3 ± 0.03 (mean ± SD) ng/g wet wt in the rat thymus and between 0.09 ± 0.01 and 0.88 ± 0.11 ng/g wet wt in the human samples]. No significant correlation was found between the levels of thymic SP-LI and the age of the patients. The highest intrathymic SP-LI content was found in a sample obtained postoperatively from an 8-month-old boy.

Chromatographic Description of SP-LI in Thymic Extracts

Combined reverse phase HPLC/RIA analyses of SP-LI in thymic extracts showed a major peak (60–70% of the total immunoreactivity) that coeluted with an authentic SP standard (Fig. 5). The remaining peak (30–40%) eluted slightly later than synthetic NKA and at a position corresponding to authentic SP sulfoxide standard. Similar profiles were obtained after analyzing SP-LI detected by antiserum SP2 (data not shown).

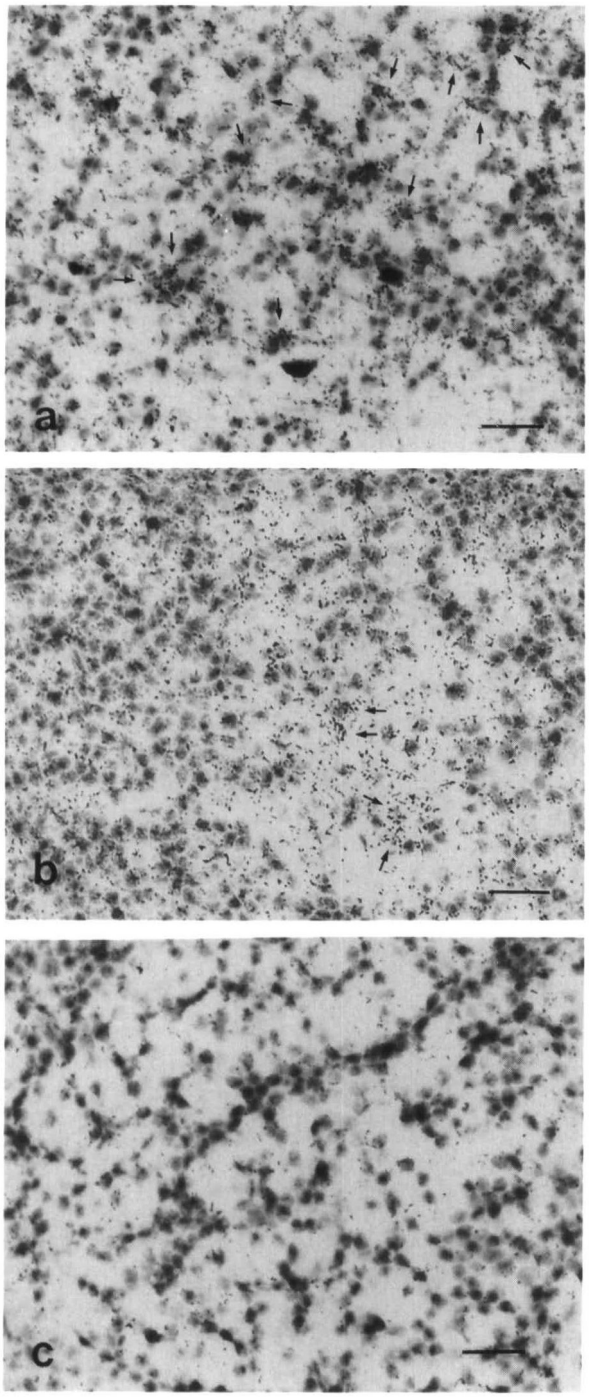

Fig. 3. Emulsion Autoradiographs of Sections from Rat Thymus

Sections from adult Sprague-Dawley rat thymus were hybridized to the PPT-A probe (a), the NPY probe (b), and the control NI-2 probe (c). Scattered hybridization-positive cells (*arrows*) can be seen over the thymic medulla in a and b. Also note the absence of labeling over any cells in the thymus in c, included as a negative hybridization control. *Scale bars* are 60 μm.

Detection of Tachykinin-LI in Rat Thymic Cells

Sections from rat thymus were immunostained using either a polyclonal SP antiserum or a polyclonal NKA antiserum. No specific immunostaining was seen with the SP antiserum (data not shown). However, the NKA antiserum revealed a specific immunostaining of stromal cells throughout the thymic parenchyma, with a predominance in the medullary area (Fig. 6). Double immunoflourescence analysis was performed with a A2B5-specific antiserum as well as KL 4 monoclonal antibodies against human cytokeratin. NKA-LI-containing cells were also specifically stained with the A2B5 antiserum and contained cytokeratin-LI, demonstrating their epithelial nature (data not shown). All NKA-LI in the rat thymus was abolished after absorbtion of the antiserum to a synthetic NKA peptide (data not shown).

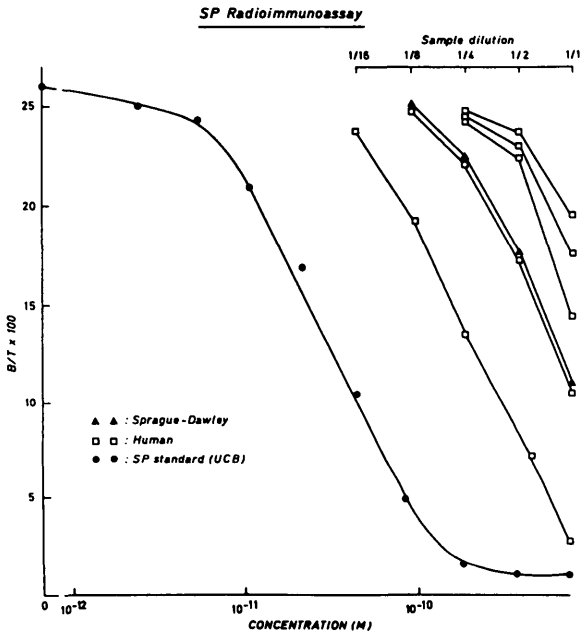

Fig. 4. Detection of SP-LI in Rat and Human Thymuses

Shown in the figure is a standard curve from the SP RIA using the SPA antiserum, as well as dilution curves of extracts prepared from five different human thymuses and a pool of six rat thymuses also using the SPA antiserum.

DISCUSSION

The results reported here confirm the recent observations describing the presence of SP-LI in the rat thymus (30) and, in addition, demonstrate the existence of this neuropeptide in the human thymic gland. Moreover, RNA blot analyses were used to demonstrate a local synthesis of PPT-A mRNA in the rat thymus. In the rat brain three different forms of PPT-A mRNA of similar length (α, β, and γ), generated by differential RNA processing, have been detected (32). Both SP and NKA are encoded by the β and γ forms, whereas the α form only encodes SP. The cDNA probe used in this study detects all three forms of PPT-A mRNA, but due to their similar size the results of the Northern blot analyses

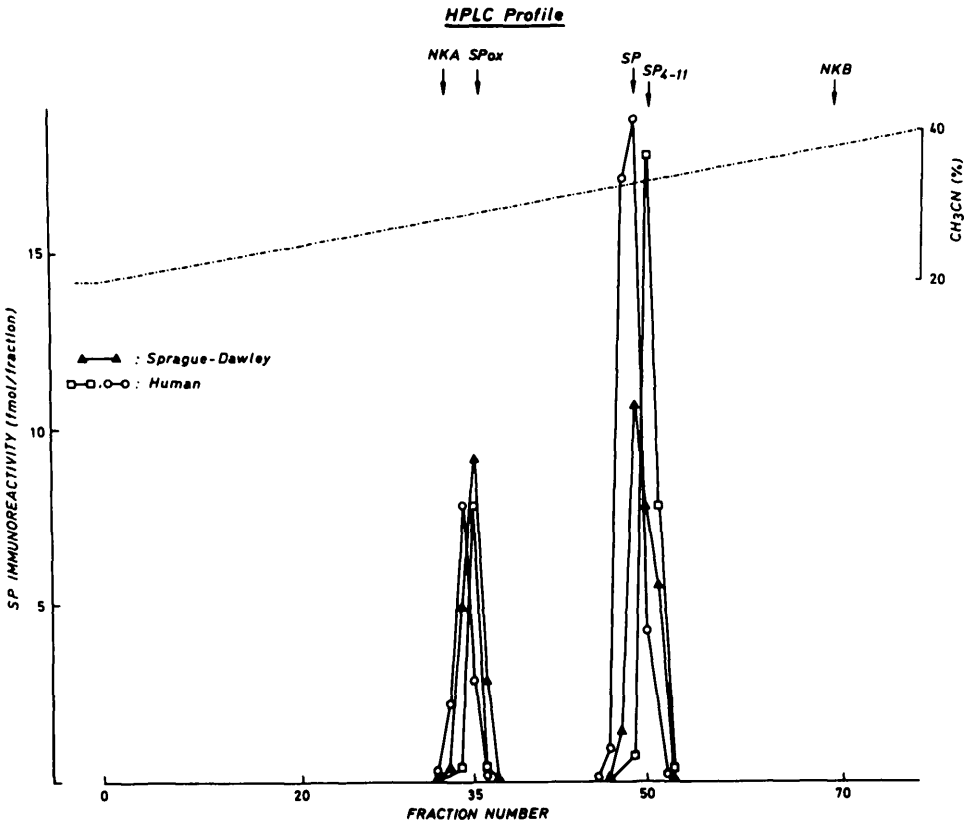

Fig. 5. Reverse Phase HPLC Profile of SP-LI Contained in Rat Thymus and Different Human Thymic Extracts

The *arrows* denote the elution positions of synthetic SP, SP-(4–11), NKA, and NKB run on the same column after analysis of the thymic extracts.

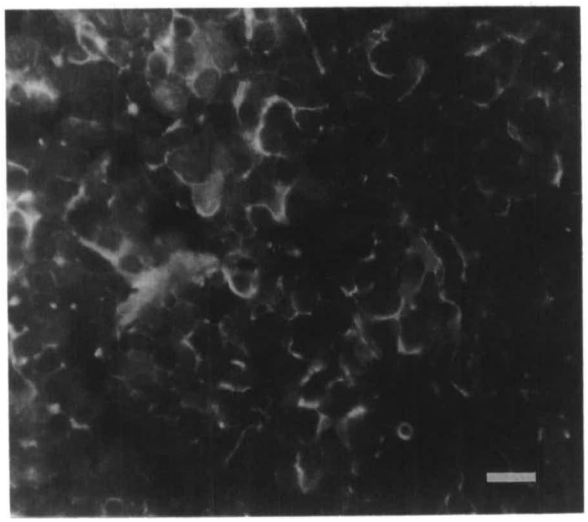

Fig. 6. Immunofluorescence Micrographs of Rat Thymus after Incubation with NKA Antiserum

Specific immunostaining can be seen in the thymic medulla with an epithelial cell-like morphology. *Bar* is 16 μm.

cannot be used to determine if all three forms or only one or two of the forms are expressed in the rat thymus.

In situ hybridization analyses demonstrated the presence of low levels of PPT-A mRNA in cells located predominantely in the thymic medulla. However, only a minor fraction of the cells in the medulla was labeled, suggesting that PPT-A mRNA expression is restricted to a small subpopulation of thymic medulla cells. Alternatively, the gene may be expressed in a larger population of thymic medulla cells, but only during a restricted time interval. An identification of the cell types expressing PPT-A mRNA in the rat thymic medulla would require combined *in situ* hybridization and immunohistochemical staining on the same section, using antisera directed against antigenic markers specific for different cell types in the thymus. However, the absense of detectable PPT-A mRNA in rat spleen and isolated rat spleen cells suggests that PPT-A mRNA is not expressed in mature T-cells. Also, the thymic nurse cells located in the outer thymic epithelium that synthesize OT and VP (9, 15, 22) are less likely to be the site of PPT-A mRNA synthesis in the thymus, given the almost complete absense of labeling in the thymic cortex. Alternative sites could be a subset of the thymic epithelial cells that express neuroendocrine antigenic markers (13–17, 36) as well as different peptides (6–9, 18, 36). In support of this, NKA-LI was deteceted in rat thymus in cells with a morphology resembling thymic epithelial cells. Surprisingly, however, immunostaining of rat thumus with a SP-specific antiserum showed no SP-LI-containing cells in the rat thymus. This may be

due to an inability of the SP antiserum to detect SP peptides in fixed tissue sections from the thymus or may be explained if PPT-A mRNA in the rat thymus is only translated into NKA peptides. Interestingly, Geppetti *et al.* (31) described and charaterized, by combined HPLC-RIA, NKA-LI in the rat thymus and showed that it was less sensitive to capsaicin than SP-LI. Hence, NKA peptides may be locally translated from the PPT-A mRNA in the thymus, which is further supported by our demonstration of NKA-immunoreactive cells in the rat thymus.

A hybridization pattern similar to the one for PPT-A mRNA was seen using a rat NPY probe. NPY mRNA and peptide have previously been detected in rat megakaryocytes and thrombocytes, and low levels of NPY were also detected in rat thymus by RNA blot analysis (37, 38). The finding of NPY mRNA in a subset of thymic medulla cells provides another example of a nonneuronal expression of NPY mRNA in the rat and suggests that NPY may serve a function in the thymus similar to that of tachykinin peptides.

A biochemical characterization of the SP-LI detected in the rat and human thymus using reverse phase HPLC revealed that 60–70% of SP-LI was identical to authentic SP. Hence, a major fraction of the intrathymic SP corresponds to the biologically active form found in nervous tissues. The remaining SP-LI did not correspond to synthetic SP. The very low cross-reactivity of the antisera used in this study with NKA or NKB strongly suggests that this additional peak was not NKA or NKB, but most likely represented the oxidized form of SP which eluted off the column at this position. Capsaicin treatment has previously been shown to decrease the level of SP-LI in rat thymus (30), suggesting that the previously detected SP-LI was at least in part derived from sensory nervous structures innervating the rat thymus. The relatively low amount of SP-LI detected in both the rat and human thymus together with the inability to detect SP-LI in thymic cells suggest that the major part of SP-LI described in this study is derived from sensory innervation of the thymus.

Specific high affinity binding sites for SP have been described in the rat thymic medulla, in association with vascular structures (39). Therefore, SP may function as a paracrine factor, perhaps regulating the local blood flow. Alternatively, tachykinin peptides, locally synthesized within the thymus, may play a role in the immune system. Previously, SP has also been shown to stimulate the mitotic activity of cultured human T-cells (33) and induce the release of cytokines from human peripheral blood monocytes (40); recently, NKA has been shown to stimulate proliferation of murine thymocytes in culture (41). Both SP and NKA have also been shown to stimulate differentiation of human B-lymphocytes into immunoglobulin-producing cells (42). In agreement with these biological activities, specific receptors for SP have been detected on macrophages and human T-cell populations (34, 43). Hence, the local synthesis of tachykinin peptides within rat and possibly human thymus together with the previous demonstrations of specific SP receptors on immunocompetent cells suggest that they may play a role in the process of T-cell differentiation and/or early activation that occurs primarily in the thymus.

MATERIALS AND METHODS

RNA Preparation and Blot Analysis

The indicated organs were dissected from adult (150–200 g BW) Sprague-Dawley rats. All tissues were frozen in liquid nitrogen and stored at −70 C before RNA preparation, as previously decribed (44). Briefly, the frozen tissue samples were homogenized in 4 M guanidine isothiocyanate, 0.1 M β-mercaptoethanol, and 0.025 M sodium citrate (pH 7.0) and centrifuged at 15 C in a Beckman SW41 rotor (Palo Alto, CA) at 35,000 rpm for 16 h. Poly(A)$^+$ RNA was isolated by oligo(dT)-cellulose chromatography (45), and the recovery of RNA was quantified spectrophotometrically before use in RNA blot analysis. Poly(A)$^+$ RNA (20 μg) from each sample was electrophoresed in a 1% agarose gel containing 0.7% formaldehyde and transferred to a nitrocellulose filter. The blotted RNA was then hybridized to a 300-basepair (bp) *Bgl*II-*Bst*EII fragment from a rat PPT cDNA clone kindly provided by Dr. Shigetada Nakanishi, Kyoto University, Japan. This fragment hybridizes to a 1.3-kb PPT mRNA encoding either SP alone or both NKA and SP (32). For hybridization to NPY mRNA, a 287-bp *Xba*I-*Ava*I fragment containing exon 2 of the NPY gene was used (35). The fragments were labeled with [α-^{32}P]dCTP by nick translation to a specific activity of approximately 5×10^8 cpm/μg. Hybridization was carried out in 4 × SSC (1 × SSC is 150 mM NaCl and 15 mM sodium citrate, pH 7.0), 40% formamide, 1 × Denhardt's solution, and 10% dextran sulfate at 42 C. Filters were washed at high stringency (0.1 × SSC-0.1 sodium dodecyl sulfate; 54 C) and exposed to Kodak XAR-5 films (Eastman Kodak, Rochester, NY). The same filters as those used for PPT hybridization were boiled for 5 min in 1% glycerol and then probed with a nick-translated 1.5-kb *Pst*I fragment encoding mouse α-actin (46). Appropriate exposures of all autoradiograms were quantified using a Shimadzu (Kyoto, Japan) CS-9000 densitometer. The level of PPT mRNA was then normalized relative to the level of actin mRNA.

In Situ Hybridization

In situ hybridization was carried out essentially as described by Ernfors *et al.* (44), with some minor modifications. Briefly, pieces of rat thymus were fresh frozen, and 14-μm thick sections were cut on a cryostat and thawed onto poly-L-lysine (50 μg/ml)-pretreated slides. The sections were subsequently fixed in 4% paraformaldehyde for 30 min and rinsed twice in PBS. The sections were then acetylated in 0.1 M triethanolamine, pH 8.0, containing 0.25% acetic anhydride for 10 min. After rinsing in 2 × SSC, followed by one rinse in 1 × PBS, the sections were immersed in 0.1 M Tris-HCl (pH 7.0) and 0.1 M glycine for 30 min. Dehydration was carried out in a graded series of ethanol, including a 5-min incubation in chloroform, followed by a brief air drying. The sections were prehybridized for 2 h at 42 C in 50% formamide, 4 × SSC, 1 × Denhardt's solution (0.02% polyvinylpyrrolidone, 0.02% Ficoll, and 0.02% BSA), 10% dextran sulfate, 0.25 mg/ml yeast tRNA, 0.5 mg/ml sheared salmon sperm DNA, 1% sarcosyl (*N*-lauroyl sarcosine), 0.02 M $NaPO_4$ (pH 7.0), and 0.05 M dithiothreitol. The same DNA fragments as those used for RNA blot analysis were also used to detect PPT and NPY mRNA by *in situ* hybridization. As a negative control probe, a 500-bp *Eco*RI fragment from a cDNA clone encoding the rat brain antigen NI 2 was used. This fragment contains the 3′-untranslated region of a gene expressed in the rat brain and mitogen-stimulated spleen cells, but not in rat thymus, as determined by RNA blot

analysis (Ericsson, A., G. Barbany, and H. Persson, manuscript submitted for publication). The DNA probes were labeled with [α-^{35}S]dATP by nick-translation to a specific activity of approximately 5×10^8 cpm/μg. Hybridization was performed at 42 C for 15 h in a humidified chamber with 100 μl/slide hybridization cocktail containing 1×10^6 cpm labeled probe. The slides were subsequently rinsed, washed four times for 15 min each at 55 C in 1 × SSC, and then air dried. The sections were dipped in Kodak NTB-2 photoemulsion, diluted 1:1 in water, exposed for 10 weeks at −20 C, developed, and fixed, followed by counterstaining with cresyl violet. Light- and darkfield micrographs were obtained using a Nikon Microphot-FX microscope. For darkfield micrographs, epiluminiscent UV light and a Nikon IGS filter was used to reduce tissue autofloures-cene and quenching of light over cells densely stained with cresyl violet.

Extraction of SP-LI from Thymic Tissues

Thymic lobes were dissected from children undergoing sternotomy for cardiac surgery. A pool of six thymuses was obtained from adult Sprague-Dawley rats after cervical dislocation. SP extraction was performed as previously described (47). Briefly, small pieces of thymus were placed for 10 min into boiling 0.5 M acetic acid (1:10, wt/vol) and then centrifuged at 4 C at 1600 × *g* for 10 min. Supernatants were collected, lyophilized, and stored at −20 C until further analyses. The estimated recovery of exogenously added SP in the extraction procedure was 76.5 ± 2.4%.

SP-Specific RIA

Two polyclonal SP-specific antisera were used for the RIA. The first antiserum (SP2) was kindly provided by Dr. Ernst Brodin, Karolinska Institute (Stockholm, Sweden), and this antiserum has previously been characterized and used for SP RIA, as detailed by Brodin *et al.* (48). The other antiserum (SP_A) was raised in rabbits after sc injections of SP conjugated to BSA using diflourodinitrobenzene (49). [Tyr^3]SP was radioiodinated using a slight modification of the chloramine-T method. For assays, lyophilisates were first reconstituted in 0.05 M phosphate buffer, pH 7.5, containing 0.5% BSA and 0.05% sodium azide. One hundred-microliter samples or standard SP were incubated with 100 μl SP2 (initial dilution, 1:50,000) or SP_A (initial dilution, 1:75,000) for 24 h at 40 C. One hundred microliters of ^{125}I-[Tyr^3]SP (±30,000 cpm) were then added together with normal rabbit serum (1:100), and the incubation was continued for 24 h at the same temerature. After the addition of 1 ml buffer containing 6% polyethylene glycol 6000, goat antirabbit antiserum (1:200), 0.5% Tween-20, and 0.02% inactive cellulose, the samples were incubated for 20 min at room temperature, and bound and free antigen were separated by centrifugation at 2,700 × *g* at 200 C for 15 min. The sensitivity of the assays was 3 pg/ml. Both antisera reacted to 100% with synthetic SP, fragment SP-(4–11), [Tyr^3] SP, and [Tyr^8]SP. Cross-reactivity was only 1% with fragment SP-(7–11) and less than 0.01% with fragment SP-(1–4), NKA, and NKB. Synthetic SP, NKA, NKB, SP fragments, [Tyr^3]SP, and [Tyr^8]SP were purchased from UCB-Bioproducts (Braine l'Alleud, Belgium). Intra- and interassay coefficients of variation were 6% and 8%, respectively.

Reverse Phase HPLC

Lyophilized extracts of rat and human thymuses were redissolved in 0.1% CF_3COOH-20% CH_3CN, filtered, and injected on a 5-μm Bondapak C18 column (Waters Associates, Milford, MA) equilibrated in the solvent. Elution was performed at a flow rate of 1 ml/min, with a linear gradient of 0.5% CH_3CN/min. Fractions of 0.5 ml were collected and lyophilized. The system was calibrated using UV-detectable amounts of synthetic SP, fragment SP-(4–11), NKA, and NKB. Standard preparations were run after analysis of the thymic extracts to avoid any artefactual shadowing effect.

Immunochemistry

Frozen pieces of rat thymus were sectioned (3–5 μm thick) on a cryostat, followed by fixation in 15% picric acid and 2% paraformaldehyde. After rinsing, the sections were processed for indirect immunoflourescence and incubated at 4 C with polyclonal SP2 antiserum (diluted 1:100) (48) or the polyclonal antiserum NKA2 against NKA (diluted 1:200) (48). Control experiments included preincubations of the antiserum with their homologous antigens (1 μM) as well as solid phase absorbtion of antiserum NKA2 with 3 mg NKA (Peninsula, Belmont, CA) coupled to 0.1 g CnBr-activated Sepharose 4b beads, handled as recommended by the manufacturer (Pharmacia, Uppsala, Sweden). Double immunoflourescence analyses with A2B5 crude supernatant or KL 4 antiserum were performed as previously described (15).

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