The interaction of substance P with intact lymphatic tissue was quantified and autoradiographically visualized, using slide-mounted tissue sections of rat spleen. Radiolabeled substance P binds rapidly to an apparently single class of noninteracting high affinity sites (K_d = 2.4 nmol/L; B_max = 9.4 fmol/mg protein). The ligand selectivity pattern suggests that substance P binding sites are similar to substance P receptors found in other tissues, including the brain, T lymphocytes, and macrophages. Substance P receptors are highly concentrated in the antigen-trapping spleen marginal zone, with low densities being found in the red pulp. No specific binding of radiolabel to T cell-dependent immunologic domains of the spleen is seen. The distribution of substance P receptors suggests that substance P is probably involved in the control of sensory functions of the immune system.

MATERIALS AND METHODS

Preparation of sections. As described previously, 24-μm-thick serial sections of frozen rat (adult female, Sprague-Dawley) spleens were thaw-mounted onto subbed slides, placed under vacuum in a chilled desiccator jar, stored overnight between 0°C and 4°C and assayed the next day. Protein determinations of representative samples of sections were performed.

Assay. Slides with two spleen sections mounted on each were placed in stainless steel racks and immersed in a medium of 158 mmol/L NaCl, 4.75 mmol/L KCl, 1.27 mmol/L CaCl_2, 1.42 mmol/L MgCl_2, 11 mmol/L dextrose, 15 mmol/L Tris-HCl at pH 7.4, containing 0.2 mmol/L/guanosine 5′ triphosphate (Sigma, St. Louis). Following a 30-minute incubation, sections were washed by two sequential five-minute incubations in 500 mL of 50 mmol/L Tris-HCl buffer, pH 7.4. To label SP receptors, incubations were performed in an assay medium consisting of 50 mmol/L Tris-HCl buffer, pH 7.4, 50 μg/mL bacitracin, 4 μg/mL chymostatin, 4 μg/mL leupeptin, 5 mg/mL bovine serum albumin (all from Sigma, St. Louis), 3 mmol/L MgCl_2, and 50 mmol/L iodinated Bolton-Hunter-coupled SP ([125]BH-SP; New England Nuclear, Pittsburgh). Following incubation at room temperature, the slides were washed by four sequential two-minute incubations in ice-cold buffer containing 3 mmol/L MgCl_2. They were dried under a stream of cold air. Nonspecific binding was defined as binding in the presence of 1 μmol/L SP, and it was normally below 15% of total binding.

For biochemical experiments, individual sections were scraped off slides and bound radioactivity was determined by gamma counting (efficiency 0.79%). For autoradiography, the sections were fixed with hot formaldehyde vapor; slides were placed in X-ray cassettes and exposed to tritium-sensitive film ([125]Ultragel; LKB, Rockville, MD). Films were developed after five days of exposure. Slides were then defatted and dipped in liquid emulsion (NTB-2; Kodak, Rochester, NY), exposed for 14 days at 4°C, developed, and counterstained with hematoxylin-eosin.

RESULTS

Characterization of [125]BH-SP binding to slide-mounted sections. The time course of association of [125]BH-SP was assessed in two separate experiments by incubation of duplicate slides (two spleen sections mounted on each) with 0.1 nmol/L [125]BH-SP for 3 to 120 minutes. At room temperature, binding reached a plateau within approximately 45
minutes, being half-maximal at 12 minutes with a pseudo-first order rate constant $K_{obs}$ of 0.055 $\text{min}^{-1}$ (Fig 1A). Incubation time of further experiments was 40 minutes.

Figure 1B illustrates that specific binding appeared to be saturable. The lower inset of Fig 1B shows a double-reciprocal plot of the specific binding data yields a straight line ($r = 0.95$), suggesting that binding occurs to a single class of sites. From this plot a $K_d$ of 2.4 nmol/L and a $B_{max}$ of 9.4 fmol/mg protein were calculated. $[^{25}I]BH-SP$ appeared to bind to noninteracting sites because a Hill plot of the specific binding data (Fig 1, upper inset) yielded a line with a slope of 0.93 ($r = 0.94$).

SP is highly potent in binding competition experiments against $[^{25}I]BH-SP$, and SP[3-11] possesses appreciable activity (Fig 2). Mean concentrations ($n = 3$) displacing 50% of specifically bound $[^{25}I]BH-SP$ (IC$_{50}$) were $0.55 \times 10^{-8}$ mol/L and $0.80 \times 10^{-6}$ mol/L, respectively. In contrast, similar concentrations of SP[1-4] failed to significantly inhibit the binding.

**Autoradiography.** The regional distribution of SP receptors within the spleen was investigated using in vitro autoradiographic techniques. SP receptors are concentrated in the red pulp (Fig 3A). Highest grain densities are found in the marginal zone (Figs 3A, 3C, and 3D). Controls treated with $10^{-9}$ mol/L unlabeled SP show minimal binding (Figs 3B, 3E, and 3F). No specific binding to structures of periarteriolar lymphoid sheath or peripheral white pulp can be seen.

**DISCUSSION**

The present biochemical results clearly indicate the presence of SP receptors in rat spleen. $[^{25}I]BH-SP$ bound with high affinity to a single class of noninteracting receptors (Fig 1). The affinity is compatible with that of $[^{25}I]BH-SP$ binding to SP receptors in rat brain slices and on synaptosomal membranes. Specificity of this high-affinity binding has been confirmed by obtaining the appropriate relative potencies of SP, SP[3-11], and SP[1-4] in competing with radiolabeled SP (Fig 3). As described for SP receptors in rat brain, SP[3-11] is about 70 times less effective in $[^{25}I]BH-SP$ binding inhibition than SP. The importance of the carboxyterminal amino acids of SP for binding is indicated by the relative weakness of SP[1-4] in displacing radiolabel. The data are in agreement with results obtained for human circulating T lymphocytes, and guinea pig peritoneal macrophages.

Autoradiographic visualization of $[^{25}I]BH-SP$ binding sites shows that SP receptors are distinctively distributed (Fig 3). Specific grains are present in red pulp. Highest grain densities are located to the marginal zone (MZ). Surprisingly, no specific binding was visible in T lymphocytic domains of the spleen, ie periarteriolar lymphoid sheath, even though 7,000 receptors per cell were demonstrated on subpopulations of human peripheral blood T lymphocytes. Direct and/or indirect evidence has been obtained for the presence of SP receptors on several types of immune cells, including T lymphocytes, B lymphocytes, polymorphonuclear leukocytes, monocytes, and macrophages. From this we conclude that the functional significance of SP receptors in the immune system depends on the physiological context.
Fig 3. Autoradiographs of [125I]BH-SP receptor distribution in rat spleen. Slide-mounted sections were incubated at optimum conditions in 0.1 nmol/L [125I]BH-SP in the absence (A, C, D) or presence (B, E, F) of 1 μmol/L unlabeled SP. (A) Distribution of grains on fixed sections without counterstain: magnification 5×. (B) Control for (A). (C) Higher magnification (×400), counterstained with hematoxylin-eosin and (D) corresponding darkfield photomicrograph. (E, F) Control for (C, D).
The MZ at the border of white pulp and red pulp of the spleen is a unique structure. Terminal branches of the arterial system in the white pulp discharge their contents between the cells of the MZ, in order for the cells to meet antigens arriving in the spleen. The antigen is picked up by a subpopulation of red pulp mononuclear phagocytes, so-called “marginal metallophyls,” and transported into the white pulp to initiate specific immune responses. Thus, the presence in high concentration of SP receptors in the MZ indicates potential importance of SP in mediation of the immune systems afferent limb function; enhancing effects of SP on phagocytosis, cell migration, and antibody production have been reported in vitro. Utilizing autoradiography, we are therefore able to correlate SP receptor expression with a functional immunologic domain in intact lymphatic tissue.

Based on the data presented in this report, no direct conclusion for the cell type that is bearing SP receptors can be drawn. Indirect evidence suggests that specialized splenic macrophages/marginal metallophyls might display SP receptors: (1) distribution patterns for SP receptors (Fig 3) and marginal metallophyls which are typically and most abundantly present in MZ appear to be identical, and (2) other cell types that are normally found in MZ, ie reticular fibrocytes, condensed sinus lining cells, thrombocytes, granulocytes, and T and B lymphocytes, are found in other spleen areas as well where no visible [125]BH-SP labeling occurred. Previously, the presence in the MZ of a subpopulation of nonrecirculating B cells has been proposed that theoretically might expose SP receptors, but in recent studies no special relationship between antibody forming cells and MZ macrophages was found.

In conclusion, the present results and those of an earlier study on receptors for vasoactive intestinal peptide show that neuropeptides possess recognition molecules in spleen which are distinctively distributed. These findings may provide a biochemical basis for the increasingly realized selective modulation of immune functions by neuropeptides.

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