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Identification and pharmacological characterization of somatostatin receptors in rat lung

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1 [^{125}I]-[LTT]SRIF-28 and [^{125}I]-SMS 201-995 were used to identify and characterize somatostatin (SRIF) receptors localized in rat lung tissue. *In vitro* autoradiography of rat lung tissue sections showed the existence of specific, high affinity binding sites for [^{125}I]-[LTT]SRIF-28 without any significant specific binding of the sst_2/sst_5-receptor selective ligand [^{125}I]-SMS 201-995.

2 In radioligand binding studies, specific binding of [^{125}I]-[LTT]SRIF-28 to membranes of rat lung was linearly related to the concentration of membrane protein used with only a small portion of nonspecific binding. With [^{125}I]-SMS 201-995 no specific binding could be observed up to a membrane concentration of 0.1 mg of protein/assay tube.

3 [^{125}I]-[LTT]SRIF-28 bound rapidly to rat lung membranes with an apparent association rate constant (k_{app}) of 1.8 ± 0.1 h^{-1} ($n=3$). The equilibrium of specific binding was reached after an incubation period of approximately 90 min at room temperature and remained constant for the next 3 h. The association rate constant (k_1) was calculated to be 3.7×10^{10} M^{-1} h^{-1}. The dissociation reaction followed first order kinetics with a dissociation rate constant (k_{-1}) $= 0.44 \pm 0.07$ h^{-1} corresponding to a half-time of 95 ± 15 min ($n=3$). From these kinetic experiments an equilibrium dissociation constant (K_D) for the binding of [^{125}I]-[LTT]SRIF-28 was calculated to be 11.9 pM.

4 Saturation binding of [^{125}I]-[LTT]SRIF-28 revealed an equilibrium dissociation constant (K_D) of 50.1 pM ($pK_D = 10.3 \pm 0.1$; $n=3$) and a receptor density (B_{max}) of 78 ± 3 fmol mg^{-1} protein. A Hill coefficient not significantly different from 1 indicated saturable binding to a single class of high affinity binding sites.

5 Specific binding of [^{125}I]-[LTT]SRIF-28 to rat lung membranes was inhibited by SRIF-14, SRIF-28 and different SRIF analogues. SRIF and different synthetic short chain SRIF analogues exhibited the following rank order of potency: SRIF-28 > SRIF-14 > CGP 23996 > > RC 160 > BIM 23014 > SMS 201-995 > BIM 23056 > MK 678.

6 The binding affinities for SRIF and the various SRIF analogues determined using rat lung tissue were in close correlation to those obtained with Chinese hamster ovary (CHO) cells stably expressing sst_1 ($r=0.92$) and sst_4($r=0.95$) receptors, respectively.

7 Reverse transcriptase - polymerase chain reaction (RT-PCR) showed the predominant expression of mRNA specific for sst_4 receptors as well as some weak sst_1 mRNA expression.

8 The findings suggest that sst_4 receptor expression is the predominant form of the somatostatin receptors identified in rat lung tissue. In this study we demonstrated for the first time the existence of sst_4 receptors in mammalian tissue.

Keywords: Somatostatin; somatostatin receptors; sst_4 receptor subtype; SRIF analogues; rat lung

Introduction

Somatostatin (somatotropin-release inhibiting factor, SRIF) is a hormone and neuropeptide that is processed into two main forms which consist of either 14 or 28 amino acids, referred to as SRIF-14 and SRIF-28, respectively (Reichlin, 1983). SRIF is an important regulator of endocrine and exocrine secretion. In addition SRIF and its stable analogues, such as SMS 201-995 (octreotide) have antiproliferative properties (Weckbecker *et al*., 1993).

SRIF was first described to be present in ovine hypothalamus (Brazeau *et al*., 1973) but was demonstrated later to be distributed throughout the body. SRIF is found in the central nervous system, pancreas, stomach and in the gut (Reichlin, 1983; Epelbaum, 1986; Yamada & Chiba, 1989). SRIF was also demonstrated to be present in lung tissue of various species. For example, SRIF has been shown to occur in lungs from guinea-pigs (Ghatei *et al*., 1982; Barrios *et al*., 1987) and neuroepithelial endocrine cells of human lung tissue (Scheuermann *et al*., 1992). However, at present only limited information is available on the importance of SRIF for lung physiology. In foetal guinea-pig and foetal goat lungs SRIF was suggested to inhibit secretion processes and arrest lung liquid production at birth (Perks & Cassin, 1987; Perks *et al*., 1992). In a recent study SRIF was shown to modulate the cholinergic neurotransmission in the ferret trachea (Sekizawa *et al*., 1989). These authors suggested that SRIF receptors are localized on cholinergic nerves in the airway system which might contribute to the inhibitory effect found for SRIF on the salbutamol-induced bronchorelaxation of canine bronchial muscles (Tamaoki *et al*., 1994). Airway mucus secretion in the rat stimulated by substance P and neurokinin A and B is inhibited by somatostatin (Wagner *et al*., 1995a,b).

The regulatory effects of SRIF are mediated by specific membrane receptors which bind SRIF-14 as well as SRIF-28 with high affinity. SRIF receptors are expressed in somatostatin target tissues such as pituitary, pancreas, gastrointestinal tract and various kinds of tumours (Schonbrunn & Tashjian, 1978; Tran *et al*., 1985; Reubi *et al*., 1988). Recently, five different somatostatin receptor subtypes have been identified and characterized (Bell & Reisine, 1993; Bruns *et al*., 1994); these receptors are now named sst_{1-5} according to IUPHAR recommendations (Hoyer *et al*., 1995). The distribution of sst_1–sst_5 mRNAs in the brain and peripheral organs of rodents and man are tissue-specific but overlapping (Kluxen *et al*., 1992;

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Yasuda *et al*., 1992; Kaupmann *et al*., 1993; Rohrer *et al*., 1993). However, for different species significant differences in the expression pattern of SRIF receptor subtypes have been described (Yamada *et al*., 1992b; Bruno *et al*., 1993).

We have shown previously the presence of mRNA specific for sst_1 and sst_4 in human lung tissue (Rohrer *et al*., 1993). Furthermore, the predominant expression of sst_4 mRNA has also been described for rat lung tissue by Bruno and coworkers (Bruno *et al*., 1993) by use of another technique (RNase protection assay). Based on these mRNA data it was of interest to investigate whether the corresponding SRIF receptor protein is also expressed in rat lung tissue.

In the present study we performed a somatostatin receptor subtype-specific reverse transcriptase - polymerase chain reaction (RT-PCR) to evaluate the expression pattern of SRIF receptor subtype mRNA in rat lung tissue. To characterize further somatostatin receptor expression, we carried out ligand binding studies to identify and characterize the SRIF receptors present. In addition, we investigated the binding affinities of various short chain SRIF analogues for SRIF receptors expressed in rat lung tissue. These affinities were compared to those obtained from binding experiments in membranes of Chinese hamster ovary (CHO) cells stably expressing $sst_1 - sst_4$ to characterize further the SRIF receptor subtypes expressed in rat lung. Finally, the distribution of SRIF receptors in lung tissue was studied by receptor autoradiography.

Preliminary accounts of some of these findings were presented to the British Pharmacological Society (Schloos *et al*., 1995).

Methods

Expression of somatostatin receptors in CHO cells

DNA of the cloned human sst_1, sst_2, sst_3 (Yamada *et al*., 1992a,b), and sst_4 (Rohrer *et al*., 1993) was subcloned into the eukaryotic expression vector pRc/CMV (Invitrogen). The resulting constructs were transfected into Chinese hamster ovary cells (CHO-K1, ATCC CCL61) by the calcium phosphate transfection method (Sambrock *et al*., 1989). Stable transfectants were selected and maintained in Ham's F-12 medium (GIBCO, No. 041-01765M) containing 0.6 mg ml^{-1} geneticin G418 (GIBCO). CHO cells were grown in Ham's F-12 medium containing 5% (vol/vol) foetal calf serum, 2 mM L-glutamin, and 0.4 mg ml^{-1} geneticin G418.

Preparation of poly(A)$^+$-RNA

Adult rats of a Sprague-Dawley strain (Ico: OFA SD, Iffa Credo, Lyon, France) were killed by decapitation. Lungs were rapidly dissected and immediately frozen in liquid nitrogen. Approximately 200 mg of frozen tissue was pulverized, added to 2 ml of extraction buffer (4 M guanidine thiocyanate, 25 mM sodium citrate, pH 7.1, 0.6 M β-mercaptoethanol), and homogenized at 25,000 r.p.m. for 1 min with a Polytron PT1200C (Kinematica AG, Luzern, Switzerland). Four millilitres of dilution buffer ((900 mM NaCl, 90 mM trisodium citrate = 6 × SSC), 10 mM TrisHCl, pH 7.4, 1 mM EDTA, 0.25% wt/vol sodium dodecyl sulphate (SDS), 0.3 M β-mercaptoethanol) were preheated to 70°C and mixed with the homogenate and 150 pmoles of biotinylated oligo(dT) probe (5′-biotin-NH-$[T]_{25}$-3′; Promega) was added. After an incubation for 5 min at 70°C, followed by centrifugation at 9500 r.p.m. (SS34 rotor, Sorvall) for 10 min at room temperature, the supernatant was added to 0.6 ml of streptavidin-coated paramagnetic particles in 0.5 × SSC (5 mg ml^{-1} BioMag Streptavidin, Advanced Magnetics). The mixture was incubated at room temperature for 2 min. The magnetic beads were captured, the supernatant was discarded and the particles were resuspended in 3 ml of 0.5 × SSC. This washing procedure was repeated four times. Finally, poly(A)$^+$-RNA was eluted in 0.6 ml of nuclease-free water. A precipitation with isopropanol followed. The concentration of the poly(A)$^+$-RNA was determined spectrophotometrically.

Reverse transcription

To eliminate potential contamination of the poly(A)$^+$-RNA with trace amounts of genomic DNA, digestion with RNase-free DNase I was performed before the reverse transcription. Poly(A)$^+$-RNA (1 μg) was incubated for 30 min at 37°C with 0.5 units RQ1 DNase, and 20 units rRNasin (Promega) in 25 μl of 10 mM Tris HCl, pH 8.0, 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT). Afterwards, addition of 6 μl DNase-stop-mix (50 mM EDTA, 1.5 M NaAc, pH 7.0, 1% SDS) was followed by phenol/chloroform extraction and ethanol precipitation. The poly(A)$^+$-RNA was reverse transcribed into first strand cDNA by using 500 ng of random hexamer primer, 20 units rRNasin, 400 units M-MLV reverse transcriptase (BRL) and the appropriate buffer (BRL) in a volume of 40 μl. The reaction was incubated for 30 min at 37°C and 30 min at 42°C. Quality and amount of the cDNA was initially analysed by specific amplification of β-actin.

Polymerase chain reaction

A 3 μl portion of the first strand cDNA reaction mixture was subjected to PCR amplification without further purification. PCR reactions of 50 μl were carried out in MicroAmp reaction tubes in a GeneAmp PCR System 9600 (Perkin Elmer Cetus). Final concentrations of the reaction components were 50 mM KCl, 10 mM Tris HCl (pH 9.0 at 25°C), 0.1% Triton X-100, 100 pmol each of 3′- and 5′ oligonucleotide primers, 0.2 mM each of dATP, dGTP, dCTP and TTP, 1–4 mM $MgCl_2$ (depending on the primer set) and 1.5 units Taq DNA polymerase (Promega). The following conditions were used for PCR: an initial denaturing step at 95°C for 2.5 min, then 40 cycles consisting of 95°C for 20 s, 62°C for 30 s and 72°C for 90 s. The amplified products were analysed on a 1.2% agarose gel stained with ethidium bromide. Polaroid photography over an u.v. transilluminator was used to visualize and document the results. To avoid contamination, aerosol resistant pipette tips were used and PCR reactions were performed on an isolated bench. Before use, tubes and water were irradiated with u.v. Oligonucleotides used as PCR-primer: for sst_1 HS18 (5′-CTGGGATGTTCCCCAATGGCACCG-3′) and HS19 (5′-GTCGTCTTGCTCAGCAAACACG-3′), for sst_2 2A (5′-TCATCAAGGTGAAGTCCTCTGG-3′) and 2B (5′-AGA-TACTGGTTTGGAGGTCTCCA-3′) (Rohrer *et al*., 1993) which detect the sst_{2A} splice variant, for sst_3 HS35 (5′-GCCCCGCGGCATGAGCACCTGCCA-3′) and HS39 (5′-CCAGGAAGTAGAGCCCAAAGAAGGC-3′), for sst_4 HS48 (5′-ACCAACATCTACCTGCTCAACCTGG-3′) and HS49 (5′-GCATAGTAGTCCAGGGGCTC-3′), for sst_5 HS504 (5′-CCTTTCCTGGCCACGCAGAACGC-3′) and HS505 (5′-GGCCAGGTTGACGATGTTGAC-3′), and for β-actin control actin-1 and actin-2 (Rohrer *et al*., 1993). The PCR primers for sst_2, sst_4 and sst_5 were 100% rat specific, the oligonucleotides for sst_1 and sst_3 were directed towards human receptors but also detect their rat homologues. All oligonucleotides were synthesized on a 380A synthesizer (Applied Biosystems) and purified by Bio-Spin 6 (Bio-Rad) column chromatography.

Preparation of rat lung membranes

Male Sprague-Dawley rats (Ico: OFA SD, Iffa Credo), weighing 150–250 g were decapitated and lungs were removed and placed in ice-cold 10 mM HEPES buffer (pH 7.5). Connective tissue was removed and the lung tissue was minced before homogenization in 10 volumes of 10 mM HEPES (pH 7.6) containing 0.25 M sucrose, 2 mM EDTA, 15 μM bacitracin, 100 μM phenylmethyl-sulphonylfluoride (PMSF), 1 μM pepstatin and 1 μM leupeptin (buffer 1), with a Polytron homogenizer (setting 9, 2 × 20 s). The lung homogenate ob-

tained was centrifuged at $500 \times g$ for 10 min. The pellet was discarded and the supernatant recentrifuged at $30{,}000 \times g$ for 30 min. The resulting pellet was resuspended in 10 mM HEPES, 2 mM EDTA, pH 7.6, 15 μM bacitracin, 100 μM PMSF, 1 μM pepstatin and 1 μM leupeptin (buffer 2) in a teflon/glass homogenizer and passed through a fine-mesh nylon screen. After three centrifugation steps at $30{,}000 \times g$ for 30 min with resuspension of the intermediate pellet in preparation buffer, the final pellet was resuspended at a concentration of 0.5 g wet weight ml^{-1}. The membrane preparation was stored at $-70°C$ until used.

Preparation of CHO cell membranes

After removal of the culture medium the cell culture bottles were washed once with ice-cold 10 mM HEPES buffer (pH 7.6). The cells were resuspended in this buffer by scraping them off from the bottle wall with a teflon cell-scraper. Thereafter the cells were homogenized with 20 strokes in a glass-homogenizer. The homogenate was centrifuged at 4°C for 30 min at 35,000 $\times g$. The supernatant was discarded and the pellet was resuspended with HEPES buffer in the glass-homogenizer. Aliquots were frozen and stored at $-70°C$ until used.

Radioligand binding assays

Membranes of CHO cells were rapidly thawed, diluted with incubation buffer (10 mM HEPES, pH 7.6, 10 mM $MgCl_2$, 15 μM bacitracin and 0.5% wt/vol bovine serum albumin) and resuspended in a glass-homogenizer. Rat lung membranes were thawed and resuspended in the same way by use of a modified incubation buffer (10 mM HEPES, pH 7.6, 10 mM $MgCl_2$, 2 mM EDTA, 15 μM bacitracin, 100 μM PMSF, 1 μM pepstatin, 1 μM leupeptin, and 0.5% wt/vol bovine serum albumin). All binding reactions were carried out in a total assay volume of 300 μl, which consisted of 40–80 μg (CHO cells) or 4–8 μg (rat lung) membrane suspension (200 μl), approximately 20 pM of [^{125}I]-[LTT]SRIF-28 or [^{125}I]-[Try^{11}]SRIF-14 ($\sim$30,000 c.p.m., 70 μl), and different concentrations of the compound under investigation (30 μl). After incubation for 90 min at room temperature, the samples were filtered under vacuum through glass-fibre filters (Whatman GF/B) by use of a 48-wells cell harvesting device (Brandel, Gaithersburg, U.S.A.). The filters were presoaked with buffer containing 0.05% polyethylenimine when using [^{125}I]-[LTT]SRIF-28 and 1% wt/vol bovine serum albumin when [^{125}I]-[Tyr^{11}]SRIF-14 was used as the respective radioligand. Following filtration the filters were rinsed immediately with 10 ml of ice-cold buffer (10 mM Tris HCl, 150 mM NaCl, pH 7.5) to reduce futher nonspecific binding of the respective radioligand to the filters and the membranes. Bound radioactivity was determined in a gamma counter (RiaGamma 1274, LKB Wallac, Turku, Finland) at 70% counting efficiency. In all experiments, specific binding was defined as the difference between total binding and nonspecific binding in the presence of an excess (1000 fold) of the appropriate unlabelled competing ligand. In kinetic and competition binding experiments the specific binding represented 70 to 75% of the total [^{125}I]-[LTT]SRIF-28 binding to rat lung preparation. Total binding of [^{125}I]-[LTT]SRIF-28 amounted to approximately 10–15% of [^{125}I]-[LTT]SRIF-28 present in the assay. All assays were performed in triplicate and repeated as indicated in the text.

Table 1 pK_i values of somatostatin (SRIF) and short chain peptide analogues of SRIF

	pK_i ($-\log$ M)				
		CHO cells			
Compound	*Rat lung*	*sst$_1$*	*sst$_2$*	*sst$_3$*	*sst$_4$*
SRIF-14	8.9	9.4	10.1	9.6	8.7
SRIF-28	9.1	9.1	9.5	9.2	9.1
SMS 201-995	6.7	6.7	9.3	7.9	6.0
BIM 23014	7.0	6.3	9.4	7.2	6.0
BIM 23056	6.6	7.0	6.7	7.0	7.0
CGP 23996	8.8	8.4	9.1	9.4	8.6
MK 678	6.0	<6.0	10.1	7.5	5.2
RC 160	7.5	6.8	10.1	7.6	6.8

The respective pK_i values for rat lung were calculated based on competition experiments as shown in Figure 6. Competition experiments with CHO cell membranes expressing human sst_1–sst_4 receptors were performed in a similar manner (not depicted), with the exception that [^{125}I]-[Tyr^{11}]SRIF-14 was used as the radioligand whereas with lung membranes [^{125}I]-[LTT]SRIF-28 was used. Each value represents the mean of 1–4 separate experiments.

Autoradiographic studies

Autoradiographic studies were performed as previously described (Bruns *et al*., 1990). Briefly, frozen rat lung tissue was sectioned and mounted on gelatin-coated glass slides. Tissue sections were incubated at room temperature for 2 h in Tris HCl buffer (pH 7.4) containing 1% bovine serum albumin, bacitracin (40 μg ml^{-1}) and $MgCl_2$ (5 mM). Ligand concentration ([^{125}I]-[Tyr^3]SMS 201-995 or [^{125}I]-[LTT]SRIF-28) was in the range of 10–30 pM. Nonspecific binding was determined from adjacent slices by coincubation with either SMS 201-995 or SRIF-28 (1 μM). Autoradiograms were obtained by exposing the labelled sections to photographic films (Hyperfilm bmax, Amersham) at 4°C for 12–16 days.

Determination of protein concentration

Protein concentrations were determined according to Bradford (Bradford, 1976) with the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, München, FRG) with bovine serum albumin (Fluka, Buchs, CH) as reference standard.

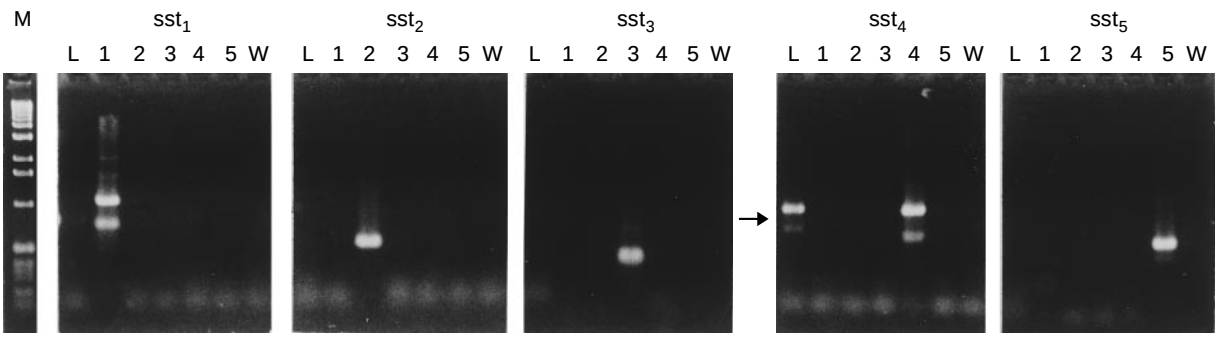

Figure 1 RT-PCR analysis of mRNA of sst_1 to sst_5 expression in rat lung tissue. The PCR templates in the different lanes used are: M = molecular weight marker 1 Kb ladder (GibcoBRL), L = rat lung cDNA, 1 to 5 = hsst_{1-5} plasmid DNA as positive controls, and W = water as negative control. The polymerase chain reaction products were separated on one 1.2% agarose gel. Expected product lengths were 909 bp for sst_1, 413 bp for sst_2, 340 bp for sst_3, 819 bp for sst_4 and 539 bp for sst_5.

Calculations

The equilibrium dissociation constants for the radioligands (K_D) and the inhibitors (K_i), maximal binding capacities (B_{max}), IC_{50} values and other binding parameters were estimated by a nonlinear least squares computer analysis on an IBM compatible computer with the ORIGIN software package (MicroCalc Software, Northampton, U.S.A.). All equations used were described in detail previously (Schloos *et al*., 1987). All values given as mean ± s.e.mean are from triplicate determinations if not stated otherwise.

Chemicals

SRIF-14, and the SRIF analogues SMS 201-995 (D-Phe-c[Cys-Phe-D-Trp-Lys-Thr-Cys]-Thr-ol), [Tyr^3]SMS 201-995, BIM 23056 (D-Phe-Phe-Tyr-D-Trp-Lys-Val-Phe-D-Nal-NH_2), CGP 23996 (c Asu-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Tyr-Thr-Ser), MK 678 (c[*N*-Me-Ala-Tyr-D-Trp-Lys-Val-Phe]), and RC 160 (D-Phe-c[Cys-Tyr-D-Trp-Lys-Val-Cys]-Trp-NH_2), where Aha is 7-aminoheptanoic acid and Nal is β-(2-naphthyl)alanine, were synthesized at Novartis Pharma (Basel, Switzerland). SRIF-28 and BIM 23014 (D-Nal-c[Cys-Tyr-D-Trp-Lys-Val-Cys]-Thr-NH_2) were obtained from Bachem (Bubendorf, Switzerland) (Table 1). [Tyr^{11}]SRIF-14 and [Leu^8,D-Trp^{22}, Tyr^{25}]SRIF-28 ([LTT]SRIF-28) were purchased from Peninsula Laboratories (Heidelberg, Germany). Na^{125}I was obtained from Amersham (Rahn AG, Zürich, Switzerland). Culture media and serum for CHO cells were obtained from GibcoBRL (Basel, Switzerland). All other chemicals were of reagent grade and purchased from commercial sources.

Radioiodination procedure

[Tyr^{11}]SRIF-14, [LTT]SRIF-28, and [Tyr^3]SMS 201-995 were radioiodinated by use of Na^{125}I (1 mCi) and chloramine-T (2–4 μg) (Hunter & Greenwood, 1963). For each SRIF analogue the labelling reaction was carried out for 1 min in 0.5 M sodium phosphate buffer (pH 7.5). The respective iodinated SRIF analogue was isolated by high-performance liquid chromatography (h.p.l.c.) on a LiChrosorb RP-18 (10 μm) reverse-phase column (244 × 4 mm, Hibar, Merck, Darmstadt, Germany). A linear acetonitrile gradient from 20 to 40% was used for separation of [^{125}I]-[LTT]SRIF-28 and [^{125}I]-[Tyr^3]SMS 201-995, and a linear acetonitrile gradient from 28 to 48% was used for separation of [^{125}I]-[Tyr^{11}]SRIF-14. The separation procedure was carried out over 40 min at a flow rate of 1.5 ml min^{-1}. The purified mono-iodinated SRIF analogues had a specific activity of about 2000 Ci mmol^{-1}.

Results

Expression of SRIF receptor subtype mRNA

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of rat lung tissue was carried out with somatostatin receptor subtype specific oligonucleotides. PCR products were separated in ethidium bromide-stained agarose gels revealing a predominant expression of the somatostatin receptor subtype sst_4 mRNA (Figure 1). Only a very weak signal for sst_1 and apparently no signals for sst_2, sst_3 and sst_5 mRNAs were obtained.

Identification of SRIF binding sites in rat lung membranes

To confirm expression of somatostatin receptors at the protein level, we performed radioligand binding experiments with [^{125}I]-[LTT]SRIF-28 and [^{125}I]-[Tyr^3]SMS 201-995 as SRIF receptor selective ligands. Specific binding of [^{125}I]-[LTT]SRIF-28 to rat lung membranes was obtained after incubation of the radioligand (25 pM) for 90 min in the absence and presence of SRIF-14 (1 μM) (Figure 2). In contrast, no specific binding of [^{125}I]-[Tyr^3]SMS 201-995 to rat lung membranes was detectable under the same conditions indicating the expression of SRIF

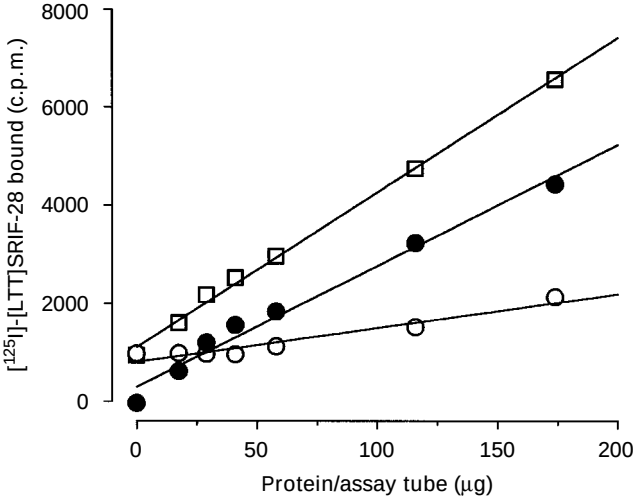

Figure 2 Protein dependency of [^{125}I]-[LTT]SRIF-28 binding to rat lung membranes. Various concentrations of rat lung membranes were incubated for 90 min at room temperature with 31.5 pM (21,500 c.p.m.) of [^{125}I]-[LTT]SRIF-28. Binding of [^{125}I]-[LTT]SRIF-28 was performed in the absence (□, total binding) and presence (○, nonspecific binding) of 1 μM SRIF-28. Specific binding (●) of [^{125}I]-[LTT]SRIF-28 was calculated as the difference between total and nonspecific binding. The straight lines are the results from linear regression analyses.

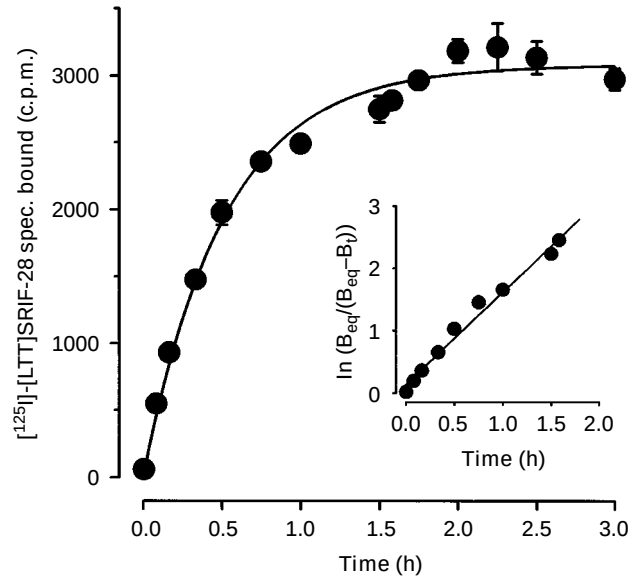

Figure 3 Time course of [^{125}I]-[LTT]SRIF-28 binding to rat lung membranes. Membranes (57.5 μg/assay tube) were incubated at room temperature with 35.4 pM (22,820 c.p.m.) of [^{125}I]-[LTT]SRIF-28 in the absence (total binding) and presence (nonspecific binding) of 1 μM SRIF-28. At the times indicated [^{125}I]-[LTT]SRIF-28 bound to the membranes was determined as described in Methods. Specific binding of [^{125}I]-[LTT]SRIF-28 as depicted was calculated as the difference between total and nonspecific binding. The line was calculated by least squares nonlinear regression analysis applying a single exponential term which gave an apparent association constant (k_{app}) of 1.94 h^{-1} corresponding to a half-time of 0.36 h. Inset: transformation of specific binding of [^{125}I]-[LTT]SRIF-28 to rat lung membranes as $\ln(B_{eq}/(B_{eq}-B_t))$ where B_{eq} is the amount of radioligand bound at equilibrium (3077 c.p.m.) and B_t is the amount of radioligand bound at the times indicated on the abscissa scale. Each point represents the mean of triplicate determinations; vertical lines show s.e.mean.

receptors that have no or very low affinity for SMS 201-995. For further investigations we therefore characterized the binding properties of [^{125}I]-[LTT]SRIF-28 to rat lung membranes in greater detail.

Protein dependency of [^{125}I]-[LTT]SRIF-28 binding to rat lung membranes

Incubation of [^{125}I]-[LTT]SRIF-28 with increasing concentrations of rat lung membranes resulted in a linear increase of total binding of the radioligand (Figure 2). The specific binding of [^{125}I]-[LTT]SRIF-28 revealed a clear linear relationship with the concentrations of rat lung membranes applied which is indicative of a specific ligand/receptor interaction. The very small linear increase of [^{125}I]-[LTT]SRIF-28 binding in the presence of an excess of unlabelled SRIF-28 (nonspecific binding) indicates, that the main part of nonspecific binding is due to an interaction of the radioligand with the glass fibre filters. In addition, [^{125}I]-[Tyr^3]SMS 201-995 was used as radioligand under the same conditions. As expected from RT-PCR and initial binding studies, no specific binding of [^{125}I]-[Tyr^3]SMS 201-995 to rat lung membranes was observed even at the highest protein concentration used (0.1 mg/assay tube).

Kinetics of [^{125}I]-[LTT]SRIF-28 binding to rat lung membranes

In order to determine the association and dissociation kinetics of [^{125}I]-[LTT]SRIF-28 binding and to establish equilibrium conditions, the time course of the interaction of [^{125}I]-[LTT]SRIF-28 with SRIF receptors expressed in rat lung was evaluated. [^{125}I]-[LTT]SRIF-28 bound rapidly to rat lung membranes with an apparent association rate constant (k_{app}) of 1.8 ± 0.1 h^{-1} ($n = 3$). A representative experiment is shown in Figure 3. The equilibrium of specific binding was reached after an incubation period of approximately 90 min at room temperature and remained constant for the next 3 h. From these experiments the time course of specific binding could be described by a monoexponential function. The association rate constant (k_1) was calculated to be 3.7×10^{10} M^{-1} h^{-1}. The dissociation of [^{125}I]-[LTT]SRIF-28 from SRIF receptors was demonstrated after addition of an excess of unlabelled SRIF-28 to block the association reaction of the radioligand (Figure 4). The dissociation reaction followed first order kinetics with a dissociation rate constant (k_{-1}) $= 0.44 \pm 0.07$ h^{-1} ($n = 3$) corresponding to a half-time of 95 ± 15 min. From these kinetic experiments an equilibrium dissociation constant (K_D) for the binding of [^{125}I]-[LTT]SRIF-28 was calculated to be 11.9 pM. Based upon these data equilibrium binding assays were performed with a 90 min incubation period at room temperature.

Saturation experiments with [^{125}I]-[LTT]SRIF-28

Under equilibrium conditions specific binding of [^{125}I]-[LTT]SRIF-28 to rat lung membranes was concentration-dependent and saturable, as demonstrated by incubating the membranes in the presence of increasing concentrations (1 to 300 pM) of [^{125}I]-[LTT]SRIF-28 (Figure 5). Transformation of the binding data obtained from saturation experiments according to Scatchard (Scatchard, 1949) resulted in a straight line indicative of an interaction of the radioligand with a single class of noncooperative sites. From three independent experiments a pK_D value of 10.3 ± 0.1 and a receptor density (B_{max}) of 83 ± 5 fmol mg^{-1} protein were calculated from nonlinear regression analysis of the saturation curves.

Competition binding experiments in rat lung membranes

Both SRIF-14 and SRIF-28 potently inhibited, in a concentration-dependent manner, binding of [^{125}I]-[LTT]SRIF-28

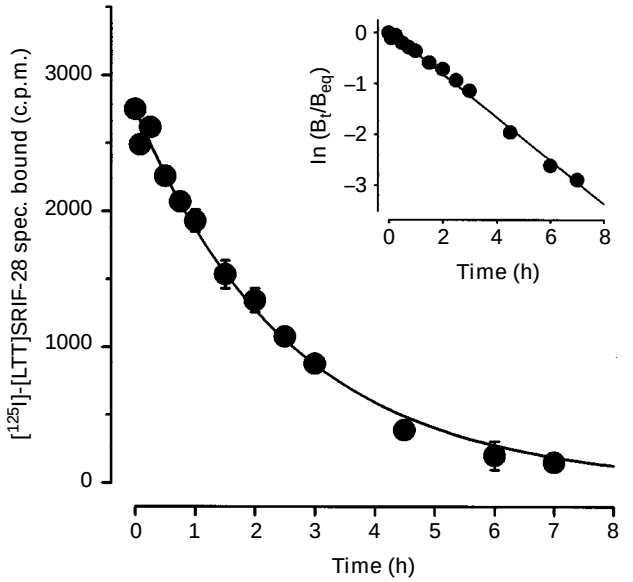

Figure 4 Dissociation time course of [^{125}I]-[LTT]SRIF-28 from SRIF receptors at rat lung membranes. Membranes were incubated with [^{125}I]-[LTT]SRIF-28 at room temperature in the absence (total binding) and presence (nonspecific binding) of 1 μM SRIF-28 and the difference was regarded as specific binding as depicted. After an incubation period of 90 min the association reaction of the radioligand was stopped by addition of 1 μM SRIF-28. Incubations were continued and at the times indicated on the abscissa scale the amount of specifically bound [^{125}I]-[LTT]SRIF-28 was determined. From nonlinear regression analysis by use of a monoexponential function the dissociation rate constant (k_{-1}) was calculated as 0.38 h^{-1} equivalent to a half time ($t_{1/2}$) of 1.82 h. Inset: transformation of specific binding data as ln (B_t/B_{eq}) where B_t refers to the amount of radioligand bound at the timepoint t indicated on the abscissa scale and B_{eq} is the amount of radioligand bound at equilibrium ($B_{eq} = 2750$ c.p.m).

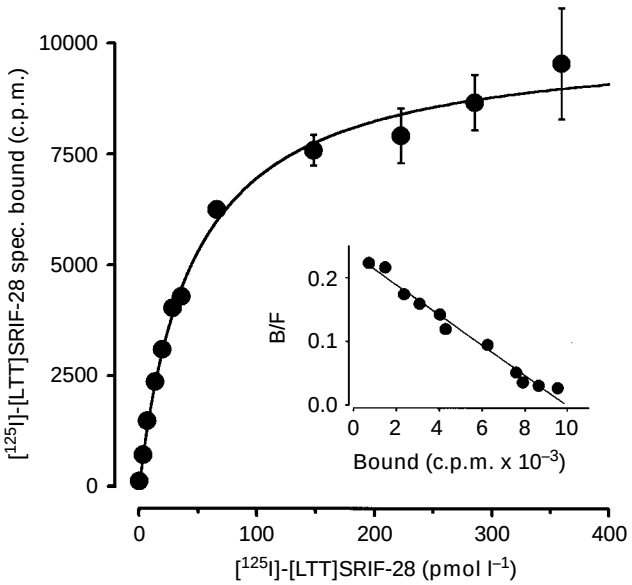

Figure 5 Concentration-dependent binding of [^{125}I]-[LTT]SRIF-28 to rat lung membranes. Membranes (57.5 μg/assay tube) were incubated for 90 min at room temperature with various concentrations of [^{125}I]-[LTT]SRIF-28 in the absence (total binding) and presence (nonspecific binding) of 1 μM SRIF-28. Specific binding of [^{125}I]-[LTT]SRIF-28 as depicted was determined by subtracting nonspecific from total binding. Nonlinear regression analysis of specific binding data gave an equilibrium dissociation constant (K_D) of 50.1 pM and a maximal binding capacity (B_{max}) of 81 fmol mg^{-1} protein. Inset: Scatchard plot analysis of specific [^{125}I]-[LTT]SRIF-28 binding to rat lung membranes. From the straight line binding of [^{125}I]-[LTT]SRIF-28 to a single class of receptors or different receptors with homogeneous affinity for the ligand can be assumed.

to rat lung membranes (Figure 6). For SRIF-14 a pK_i value of 8.9 ± 0.2 ($n=3$) and for SRIF-28 a pK_i of 9.1 ± 0.1 ($n=3$) was derived similar to those previously obtained for [^{125}I]-[Tyr^{11}]SRIF-14 binding at HEK 293 cells expressing the somatostatin receptor subtype sst_4 (Kaupmann *et al*., 1993). The SRIF-28 analogue [LTT]SRIF-28 had a slightly higher affinity than SRIF-28, demonstrated by a pK_i value of 9.4 ± 0.3 ($n=4$). Interestingly, the iodination to [^{125}I]-[LTT]SRIF-28 resulted in a significantly higher affinity ($pK_D = 10.3 \pm 0.1$, $n=3$) compared to noniodinated [LTT]SRIF-28. In order to characterize further SRIF receptors expressed in lung tissue, a variety of synthetic SRIF analogues were evaluated for their ability to compete with [^{125}I]-[LTT]SRIF-28 for binding to rat lung membranes. Representative competition curves of the SRIF analogues employed are depicted in Figure 6. All SRIF analogues caused a concentration-dependent reduction of [^{125}I]-[LTT]SRIF-28 binding with a rank order of potency of CGP 23996 > RC 160 > BIM 23014 > SMS 201-995 ⩾ BIM 23056 > MK 678. The competition curves were steep and monophasic indicative of an interaction of the compounds with homogeneous binding sites. The calculated pK_i values for these compounds are given in Table 1.

Competition binding experiments in membranes from CHO cells expressing sst_1–sst_4 receptors

In order to evaluate and to characterize the binding properties of SRIF receptors identified in rat lung, the binding profile of the various SRIF analogues was compared with the results obtained from sst_1–sst_4 binding in CHO cells expressing the respective human sst receptor subtype. CGP 23996 and BIM 23056 showed properties as universal ligands, since CGP 23996 and BIM 23056 showed no subtype selectivity and were nearly equipotent with respect to the four somatostatin receptor subtypes sst_{1-4} examined. However, the affinity of CGP 23996 at sst_{1-4} was about 100 fold higher compared to BIM 23056. All other SRIF analogues studied, SMS 201-995, BIM 23014, MK 678 and RC 160 bound with highest affinity at the sst_2 receptor subtype resulting in a rank order of affinity of $sst_2 > sst_3 > sst_4 \geqslant sst_1$. The p$K_i$ values of all compounds evaluated with respect to the human SRIF receptor subtypes are summarized in Table 1.

From the RT-PCR studies in rat lungs the expression of mRNA specific for the somatostatin receptor subtypes sst_4, and to a far lesser extent sst_1 became apparent. Therefore the affinities of the different SRIF analogues obtained from rat

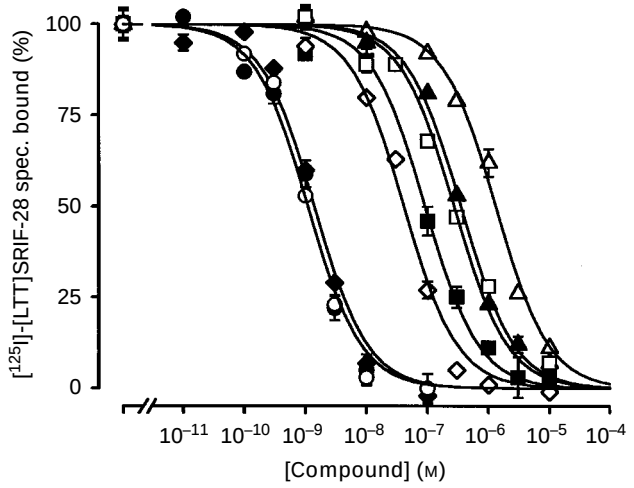

Figure 6 Competition of SRIF analogues with [^{125}I]-[LTT]SRIF-28 for binding at rat lung membranes. Membranes (approx. 80 μg) were incubated for 90 min at room temperature with 25 pM of [^{125}I]-[LTT]SRIF-28 (26,000 c.p.m.) without (control) and in the presence of the indicated concentrations of the compounds (●) SRIF-14, (○) SRIF-28, (◆) CGP 23996, (◇) RC 160, (■) BIM 23014, (□) SMS 201995, (▲) BIM 23056, (△) MK 678 or 1 μM SRIF-28 (nonspecific binding). Specific binding was calculated as the difference between total and nonspecific binding. Binding values on the ordinate scale are given as % of specific binding of [^{125}I]-[LTT]SRIF-28 obtained in the presence of the respective competitor. The lines represent the result of the computer derived non-linear regression analysis by use of an equation for non-cooperative interaction with a single class of binding sites. The K_i values calculated from the competition curves are given in Table 1. Each point represents the mean of triplicate determinations; vertical lines show s.e.mean.

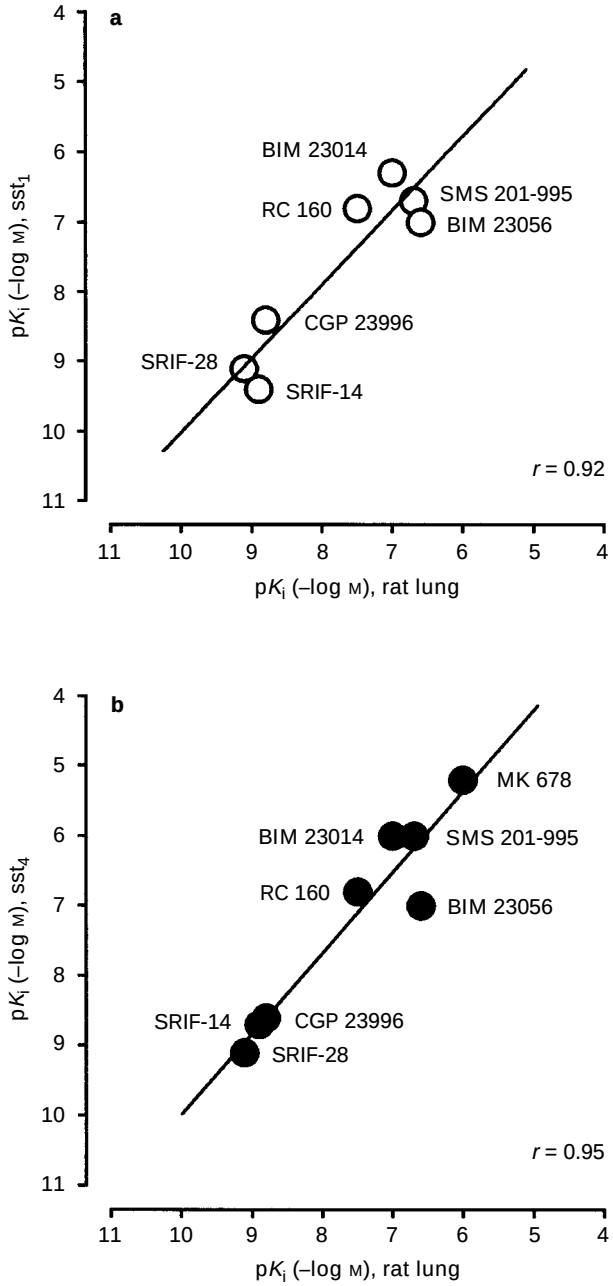

Figure 7 Correlation of the pK_i values of various compounds determined from competition experiments in rat lung membranes and sst_1 and sst_4 receptors at CHO cell membranes. pK_i values were derived from competition experiments with [^{125}I]-[LTT]SRIF-28 binding to rat lung membranes and [^{125}I]-[Tyr^{11}]SRIF-14 binding to CHO cell membranes expressing human sst_1 (a) and sst_4 (b) receptors. The pK_i values of the different compounds used are given in Table 1. The respective line in the correlations represents the result of linear regression analysis.

lung binding were compared with results from somatostatin receptor subtypes sst_1 and sst_4 binding experiments. The resulting correlations were very close and linear for sst_1 ($r = 0.92$) and sst_4 ($r = 0.95$) and are depicted in Figure 7. This characterizes the SRIF receptors in rat lung as sst_1 and/or sst_4 somatostatin receptor subtypes.

Demonstration of specific [^{125}I]-[LTT]SRIF-28 binding sites in rat lung tissue by autoradiography

To characterize further the somatostatin binding sites identified in lung tissue, we performed *in vitro* autoradiographic studies with tissue sections from rat lung with [^{125}I]-[LTT]SRIF-28 and [^{125}I]-[Tyr^3]SMS 201-995 as specific ligands. As can be seen in Figure 8, high affinity binding of [^{125}I]-[LTT]SRIF-28 could be demonstrated by autoradiography. The binding of [^{125}I]-[LTT]SRIF-28 was displaced by unlabelled SRIF-28 and SRIF-14 indicating the specific interaction of this radioligand with SRIF receptors in lung tissue. The specific binding sites were distributed homogeneously over the lung tissue section. As expected from the PCR studies and membrane binding assays, no specific binding for [^{125}I]-[Tyr^3]SMS 201-995 could be demonstrated, since short synthetic analogues such as octreotide do not bind to the sst_1 or sst_4 receptor subtype with high affinity.

Discussion

In this present study we identified and characterized SRIF receptors expressed in rat lung tissue. Tissue distribution studies of somatostatin receptors have shown both a tissue-specific and overlapping expression of receptor subtype mRNAs in various brain regions and peripheral organs of rodents (Kluxen *et al*., 1992; Yasuda *et al*., 1992; Bruno *et al*., 1993; Kaupmann *et al*., 1993) and man (Yamada *et al*., 1992a; Rohrer *et al*., 1993). Interestingly, multiple SRIF receptor genes can be coexpressed within the same cell (Patel *et al*., 1994b). However, significant species related differences have been found for the expression of SRIF receptor subtypes upon investigation of various tissues from rodents and man (Yamada *et al*., 1992b; Bruno *et al*., 1993). In our study, the examination of the distribution of mRNAs specific for the five cloned SRIF receptors suggested that in rat lung tissue two SRIF receptor subtypes, predominantly the sst_4 receptor subtype and to a much lesser extent the sst_1 subtype, are expressed. The presence of mRNA for sst_4 in rat lung tissue has also been shown by Bruno and coworkers (Bruno *et al*., 1993). In addition, these authors described the expression of sst_3 mRNA in rat lung which was not found in the present study. However, in both studies the signal for sst_4 mRNA was predominant. The predominant expression of sst_4 mRNA as well as lower levels of sst_1 mRNA has previously been found for human lung (Rohrer *et al*., 1993), indicating, that there might be only small species related differences for expression of SRIF receptors in lung tissue.

As demonstrated by our radioligand binding studies and receptor autoradiography, the binding of [^{125}I]-[LTT]SRIF-28 to somatostatin receptors of rat lung was saturable and of high affinity. For a further characterization of these SRIF receptors different short chain SRIF analogues were used in the binding experiments (Bruns *et al*., 1996). The native peptides SRIF-14 and SRIF-28 bound with similar high affinity to rat lung tissue, as also shown for the SRIF receptor subtypes $sst_1 - sst_4$ (Yamada *et al*., 1992a; Raynor *et al*., 1993a,b). In contrast the sst_5 receptor subtype was shown to exhibit an 8–30 fold greater affinity for SRIF-28 than for SRIF-14 (O'Carroll *et al*., 1992; Patel *et al*., 1994a). Thus, the rat lung SRIF receptor is clearly pharmacologically different from the sst_5 subtype. In addition, RT-PCR analyses confirmed the absence of sst_5 in rat lung tissue. Furthermore the lack of specific [^{125}I]-[Tyr^3]SMS 201-995 binding, as demonstrated by autoradiography (Figure 8), rules out a significant expression of sst_2 and sst_5 receptors in rat

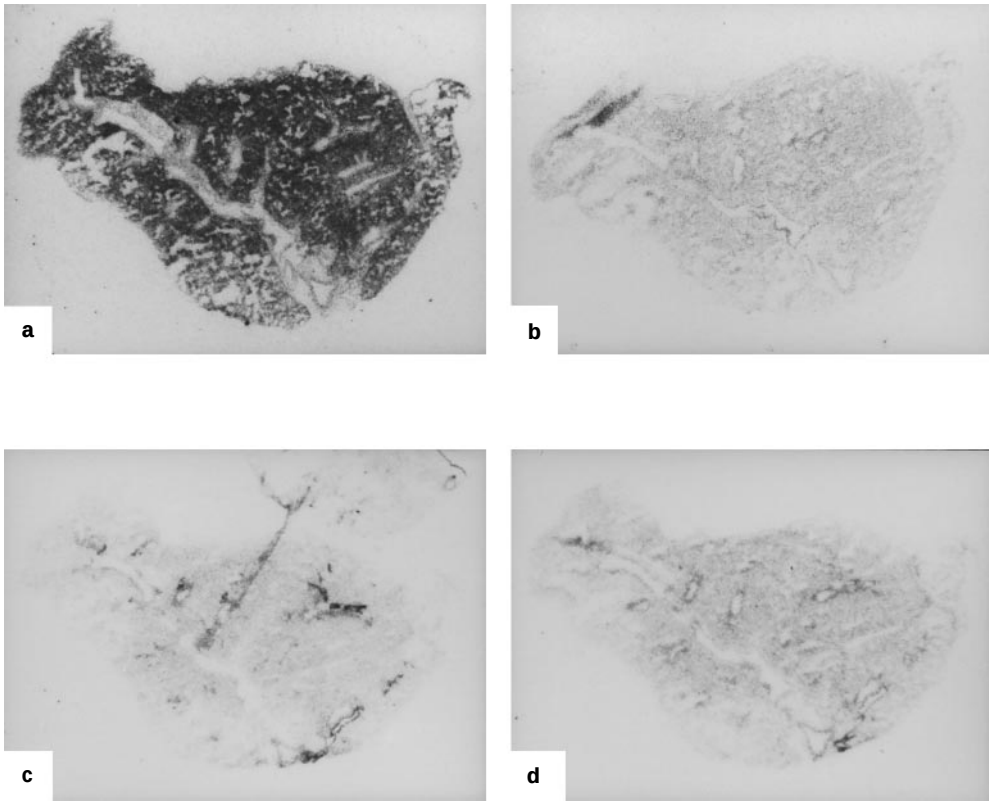

Figure 8 Autoradiographic studies in rat lung tissue. (a) Total binding of [^{125}I]-[LTT]SRIF-28 to a section of rat lung tissue. (b) Distribution of nonspecific binding of [^{125}I]-[LTT]SRIF-28 which was determined by coincubation with 1 μM SRIF-28. (c and d) Binding of [^{125}I]-[Tyr^3]SMS 201-995 as a specific sst_2/sst_5 receptor radioligand in the absence (c) and presence (d) of 1 μM SMS 201-995 to sections of rat lung tissue.

lung tissue, since SMS 201-995 shows high affinity binding to these SRIF receptor subtypes. To discriminate further between sst_1, sst_3 and sst_4 receptor expression in the rat lung we used various SRIF analogues in competition binding experiments. For a comparison binding studies were performed with membrane preparations from CHO cells expressing the respective human sst_{1-4} receptor subtype. Human and rat SRIF receptor subtypes show nearly identical binding characteristics except for the sst_5 receptor subtype, since analogues of the octreotide family bind to the rat sst_5 receptor with significantly higher affinity compared to the human sst_5 receptor (Bruns *et al*., 1995). In the present study, SMS 201-995 and MK 678 exhibited a more than 10 fold lower affinity to the SRIF receptors in rat lung compared to the sst_3 receptor subtype, suggesting that sst_3 receptor protein does not contribute significantly to the SRIF receptors expressed in rat lung tissue. Thus, from the binding studies the SRIF receptors identified in rat lung tissue exhibited binding properties closely correlated with the human sst_1 and sst_4 receptor subtypes stably expressed in CHO cells.

Although sst_1/sst_4 selective compounds are not available at present as they are for sst_2, sst_3 and sst_5 receptors (Raynor *et al*., 1993a,b; Castro *et al*., 1996), our results clearly show that SRIF receptors in rat lung belong to the sst_1/sst_4 subtype family. For further functional investigations of the role of SRIF and SRIF receptor subtypes in rat lung tissue, sst_1 and sst_4 selective ligands would be of advantage, as would be SRIF receptor subtype-specific antibodies for immunohistochemistry. A major challenge will be the determination of the specific cell types exhibiting functional SRIF receptors in lung tissue.

At present only limited information is available about the role of SRIF in lung physiology. Lung mast cells secrete histamine in response to a variety of stimuli, including somatostatin (Tomioka *et al*., 1989). SRIF-14 and SRIF-28 were demonstrated to be present in lung tissue from perinatal guinea-pigs where these peptides diminish fluid production during the onset of breathing (Perks *et al*., 1992). Furthermore, somatostatin-like immunoreactivity has also been found in the lung from a series of adult mammals (Ghatei *et al*., 1982), foetal monkeys (Dayer *et al*., 1985) and foetal sheep (Balaguer *et al*., 1992). Since the levels of somatostatin-like immunoreactivity fall immediately in the first hours after the commencement of breathing, SRIF might contribute to this early regulatory function. Another reason for the decline of somatostatin could be due to enzymatic degradation which occurs in adult lung (Ruggere & Patel, 1985). Somatostatin present in lung tissue might act also as a regulator of vascular reactivity, since hypoxic vasoconstriction was prolonged in rat isolated lungs (Sakai & Voelkel, 1988).

Recently, an inhibitory effect of SRIF on salbutamol-induced bronchorelaxation in canine bronchial smooth muscle has been described (Tamaoki *et al*., 1994), which might be explained by a down-regulation of β-adrenoceptor function. However, somatostatin analogues given to patients with hepatopulmonary syndrome (either breathing room air or 100% oxygen) were without significant effect upon changes of arterial oxygen partial pressure (PO_2) (Krowka *et al*., 1993). However, the lack of effect seen in these patients might be due to the sst_2 receptor selectivity of the SRIF analogue used (SMS 201-995, octreotide), since at present no SRIF analogue with high affinity to sst_1 and/or sst_4 receptors is available for studies in man.

In summary, our results demonstrate the predominant expression of sst_4 receptors at the RNA and protein level in rat lung tissue. This extends our previous study on mRNA specific for sst_4 identified in human lung by RT-PCR and Northern blot analysis (Rohrer *et al*., 1993). The lung is one of several nonclassical SRIF target tissues where SRIF receptor expression has been demonstrated. Up to now the relevance of sst_4 expression in rat lung tissue is not known. Whether SRIF analogues, acting as agonists or antagonists at sst_1 and/or sst_4, could be useful in the treatment of airway diseases, e.g. bronchial asthma, remains to be elucidated.

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