Properties of Soluble Somatostatin-Binding Protein

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A soluble somatostatin-binding protein was detected in the cytosol fractions of various rat, human and bovine tissues. Maximum binding occurred at pH 8.0–8.5 and was Ca^{2+} -dependent. The specific binding of somatostatin per 10 μg of cytosol protein from 12 rat tissues ranged between 36 and 15%, and 3% for peripheral blood cells. There was also substantial binding in cytosol from human anterior pituitary and liver, and bovine anterior pituitary. The specific binding in rat and human plasma in the presence of EDTA was only 1%. Gel filtration suggested a molecular weight of approx. 80 000 for the somatostatin-binding protein from several sources. Exposure of the binding protein to trypsin eliminates somatostatin-binding activity but ribonuclease and deoxyribonuclease have no effect. The binding protein is thermolabile, ethanol-precipitable, and not completely specific for somatostatin. Bound ^{125}I -labelled [Tyr¹]somatostatin is not easily displaced by excess of unlabelled somatostatin. The effects of dithiothreitol and mercaptoethanol on the binding of ^{125}I -labelled [Tyr¹]somatostatin to the binding protein suggests that binding involves two sequential steps, first loose binding, then disulphide linkage. Since semipurified somatostatin-binding protein causes a dose-related inhibition of the binding of ^{125}I -labelled [Tyr¹]somatostatin in radioimmunoassays for somatostatin, estimates of somatostatin content of tissue extracts by radioimmunoassay in some cases may be spuriously high. It is not yet clear whether the binding protein is a true cytosol protein or an easily solubilized membrane protein.

Somatostatin inhibits pituitary somatotropin secretion (Brazeau *et al.*, 1973), but also affects the function of the pancreas (Koerker *et al.*, 1974), stomach (Bloom *et al.*, 1974) and brain (Renaud *et al.*, 1975; Cotzias & Papavasiliou, 1976; Havlicek *et al.*, 1976). Despite many reports about the effects of somatostatin on different organs, the physiological role and mechanism of action of somatostatin is as yet unknown. It is widely assumed that the first interaction between a hormone and its target tissue occurs at the receptor, so detection of specific binding sites for somatostatin may help identify potential target tissues for somatostatin, and also may elucidate the mechanism of action of somatostatin.

In a search for specific binding sites for somatostatin, we used ethanol precipitation to separate bound and free somatostatin in particulate fractions (Ogawa *et al.*, 1976). Subsequently, we discovered that somatostatin was bound to a soluble protein in the cytosol fraction and that ethanol

precipitates soluble binding components in the cytosol fraction.

The present study was designed to identify and characterize a soluble somatostatin-binding protein in cytosol fractions from several tissues.

Materials and Methods

Hormone and drug preparations

Somatostatin (AY-24, 910) and [Tyr¹]somatostatin, products of Ayerst Research Laboratories, Montreal, Que., Canada, were generously provided by Dr. M. Götz.

To determine the cross-reactivity, the following hormones were used. Luliberin (luteinizing-hormone-releasing factor) was provided by Ayerst Research Laboratories. [Ala³, Ala¹⁴]Somatostatin, [D-Trp⁸]somatostatin and α -endorphine were gifts from Dr. R. Guillemin (Salk Institute, La Jolla, CA, U.S.A.). Thyroliberin (thyrotropin-releasing factor) was a

gift from Takeda Chemical Industries, Osaka, Japan. [Met]Enkephalin was obtained from Peninsula Laboratories, San Carlos, CA, U.S.A., and Naloxone (*N*-allyl-nor-oxymorphone hydrochloride) was from Endo Drugs Ltd., Montreal, Que., Canada. Human somatotropin (NIH NS2019G), dog somatotropin (NIH D-1001A), ox somatotropin (NIH B-1003A), pig somatotropin (NIH P-526B), sheep somatotropin (NIH 0-743B), rat somatotropin (NIH RP-1), human prolactin (F-3), sheep prolactin (NIH P-S-10), rat prolactin (NIH RP-1), ox thyrotropin (NIH TSH-B-3), human follitropin (follicle-stimulating hormone; NIH LER 1336), human lutropin (luteinizing hormone; NIH LER 960), sheep lutropin (NIH LH-S18) and human placental lactogen were obtained from the National Institute of Arthritis, Metabolism and Digestive Diseases, National Institutes of Health, Bethesda, MD, U.S.A. Sheep placental lactogen was purified in this laboratory (Chan *et al.*, 1976). Synthetic [Lys⁸]-vasopressin and synthetic oxytocin were gifts from Sandoz, Basel, Switzerland, and pig glucagon was a gift from Eli Lilly and Co., Indianapolis, IL, U.S.A. Synthetic corticotropin and pig insulin were bought from Organon Canada Ltd., Toronto, Ont., Canada, and Connaught Laboratories Ltd., Toronto, Ont., Canada, respectively.

Dithiothreitol, trypsin, soya-bean trypsin inhibitor, RNAase* (bovine pancreas), DNAase I (bovine pancreas), phospholipase C and bovine serum albumin were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Mercaptoethanol was bought from Eastman Kodak Co., Rochester, NY, U.S.A. and guanidine hydrochloride from Schwartz/Mann, Orangeburg, NY, U.S.A. Urea, NaCNS and H₂O₂ [30% (v/v) solution] were bought from Fisher Scientific Co., Fair Lawn, NJ, U.S.A. Lactoperoxidase was from Calbiochem, La Jolla, CA, U.S.A. Na¹²⁵I (carrier-free) was purchased from New England Nuclear, Boston, MA, U.S.A. Activated charcoal (Norit A) was from Matheson Coleman and Bell Manufacturing Chemists, Norwood, OH, U.S.A. CM-cellulose (Whatman CM-23), was from W. and R. Balston Ltd., Maidstone, Kent, U.K. Sephadex and Dextran T70 were obtained from Pharmacia, Uppsala, Sweden. All other reagents and chemicals were reagent grade.

Tissue samples and preparation of cytosol fractions and plasma

Sprague-Dawley female rats (200–225 g; Biolab Inc., St. Paul, MI, U.S.A.) were killed by decapitation without anaesthesia between 09:30 and 11:00 h. Organs listed in Tables 1 and 3 were removed, quickly frozen on solid CO₂ and homo-

genized on the same day. Human pituitary glands and livers were collected from autopsy (the sex and cause of death were unknown), and were stored at –20°C for several months. Bovine pituitaries were obtained from Canada Packers, Winnipeg, Man., Canada, and stored at –20°C.

Blood samples from rats were collected by decapitation, and from humans (authors) by venipuncture. Blood samples (5 ml) were collected in chilled glass test tubes containing 50 mg of EDTA, shaken briefly and centrifuged at 2000g for 15 min at 4°C.

Tissue and peripheral blood cells were homogenized in 10 vol. (w/v) of ice-cold Tris/EDTA buffer (50 mM-Tris/HCl buffer, pH 8.0, containing 25 mM-EDTA) with a Brinkman Polytron PT-10 apparatus (setting at 7, duration 10 s, and repeated once). The homogenates and plasma were centrifuged at 200 000g for 60 min (Beckman L5-65 ultracentrifuge), and supernatants (cytosol fraction) were quickly frozen in solid CO₂ and stored at –20°C.

Protein concentrations were determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

In preliminary experiments, tissues were homogenized in ice-cold 0.3 M-sucrose followed by differential centrifugation as described previously (Shiu *et al.*, 1973; Posner *et al.*, 1974).

Gel filtration

Samples were applied to a column (1.2 cm × 50 cm) of Sephadex G-100, equilibrated with Tris/EDTA buffer at pH 8.0 and 4°C, and 2 ml fractions were collected. The column was calibrated with Blue Dextran, ¹²⁵I-labelled human somatotropin, ¹²⁵I-labelled [Tyr¹]somatostatin and somatostatin, the distribution of the latter being determined by radioimmunoassay (see below). The protein concentration in the fraction was estimated from the A₂₈₀.

Assays

Iodination. Since somatostatin lacks amino acid residues that can be iodinated, [Tyr¹]somatostatin is used for iodination. The biological activity of this analogue is reported to be 107% (Vale *et al.*, 1976) or 100% (Ferland *et al.*, 1976) compared with the native hormone. ¹²⁵I-labelled [Tyr¹]somatostatin was prepared by the lactoperoxidase method of Thorell & Johansson (1971) with several modifications. To 25 μl of 0.5 M-NaH₂PO₄ solution (pH 4.2) was added: 5 μg of [Tyr¹]somatostatin in 5 μl of 0.1 M-acetic acid, 5 μg of lactoperoxidase in 5 μl of 0.1 M-sodium acetate buffer (pH 5.6), 1 mCi of Na¹²⁵I in 25 μl of 0.5 M-phosphate buffer (pH 7.0)

* Abbreviations: RNAase, ribonuclease; DNAase, deoxyribonuclease.

and 5 μ l of 0.002% H_2O_2 . After 1 min the reaction was stopped by dilution with 1 ml of ice-cold 2 mM-ammonium acetate buffer (pH 4.6). The reactants were applied to a column (1 cm $\times$ 10 cm) of CM-cellulose equilibrated with the above buffer. Somatostatin was eluted with a continuous gradient (0.002–0.25 M) of ammonium acetate buffer at pH 4.6. The specific radioactivity of ^{125}I -labelled [Tyr^1]somatostatin ranged from 80 to 120 $\mu\text{Ci}/\mu\text{g}$.

Subcellular distribution of somatostatin-binding sites. Initially studies of somatostatin-binding sites were done by methods similar to those described for prolactin (Shiu *et al.*, 1973; Posner *et al.*, 1974), except that bound and free ^{125}I -labelled [Tyr^1]somatostatin were separated by adding cold (4°C) ethanol to a final concentration of 85% (v/v). The radioactivity in the pellet was counted in an automatic gamma counter after centrifugation at 2000g for 20 min at 4°C.

Competitive protein-binding assays (binding assay). Binding studies were conducted with the crude cytosol fraction (40 μg of protein per assay tube). Each assay tube contained 15000–20000 c.p.m. of ^{125}I -labelled [Tyr^1]somatostatin with or without 0.1 ml of a solution (10 $\mu\text{g}/\text{ml}$) of somatostatin, and with Tris/EDTA/albumin buffer (50 mM-Tris/HCl buffer, pH 8.0, 25 mM-EDTA and 0.5% bovine serum albumin) in a total volume of 0.5 ml. Samples were usually incubated at 4°C for 18 h. After the reaction time, 1 ml of chilled dextran-coated charcoal suspension (0.25% activated charcoal and 0.025% Dextran T70 in Tris/EDTA/albumin buffer) was added. This binds approx. 98% of free ^{125}I -labelled [Tyr^1]somatostatin. Tubes were agitated on a Vortex mixer and left for 30 min before centrifugation for 10 min at 2000g and 4°C. The supernatant which represents bound hormone was counted in an automatic gamma counter. Specific binding was the difference between radioactivity bound to the somatostatin-binding protein in the presence of excess of somatostatin (1 $\mu\text{g}/\text{tube}$) and that bound in its absence, and was expressed as a percentage of the total radioactivity added per tube.

Effect of various reagents and conditions on binding

Cytosol fractions from female rat liver and bovine anterior pituitaries were used. The effect of pH was studied by exposing cytosol fractions to acetic acid or NaOH (final concentration 0.1 M) for 30 min before neutralization. The effect of ethanol was examined by adding cold (4°C) ethanol to a final concentration of 85% (v/v) and reaction tubes were centrifuged at 2000g for 20 min at 4°C. The supernatant was decanted and the pellet dissolved in Tris/EDTA/albumin buffer and assayed. Temperature effects were studied by exposing tissue

cytosols to 56°C for 30 min, 100°C for 1 min, or freezing and thawing 20 times (thawing at 20°C for 20 min).

Cytosol fractions were incubated at 37°C with various enzymes listed in Table 5 for 15 min in the Tris/EDTA/albumin buffer and then chilled in ice. For trypsin digestion, five times as much soyabean trypsin inhibitor as trypsin was added. Cytosol fractions, similarly incubated, but without enzymes, were used as controls.

All samples were adjusted to 40 μg of protein per tube before assay.

Dissociation experiments

Cytosol fractions of female rat liver and bovine anterior pituitaries were incubated with ^{125}I -labelled [Tyr^1]somatostatin at 4°C for 18 h and then a dextran-coated charcoal suspension was added, followed by centrifugation at 2000g for 10 min. To the supernatant (tracer-binding protein complex), 0.1 ml of somatostatin (10 $\mu\text{g}/\text{ml}$) or Tris/EDTA/albumin buffer was added and the dissociation of ^{125}I -labelled [Tyr^1]somatostatin was examined over 24 h.

Cytosol fractions were also incubated with ^{125}I -labelled [Tyr^1]somatostatin for 1.5, 12 or 24 h at 4°C and the dextran-coated charcoal suspension was added followed by centrifugation at 2000g for 10 min. To the supernatants were added the reagents listed in Table 6, to give the final concentration indicated, and left for 30 min at 24°C. In each case, exposure to the reagent was followed by the addition of 1 ml of dextran-coated charcoal suspension and a second centrifugation at 2000g for 10 min. The radioactivity in the second pellet (free tracer) was counted on an automatic gamma-counter.

Radioimmunoassay of somatostatin

To each assay tube was added 0.2 ml of the buffer used for binding assays, 0.1 ml of anti-somatostatin rabbit serum (1:4000 dilution) prepared by the authors (Epelbaum *et al.*, 1977), 0.1 ml of somatostatin solution in concentrations from 10 pg/ml to 100 ng/ml, or samples, and 0.1 ml of ^{125}I -labelled [Tyr^1]somatostatin (15000–20000 c.p.m.). This reaction mixture was incubated at 4°C for 48 h, then 0.1 ml of 5% sheep anti-(rabbit γ -globulin) serum and 0.1 ml of 1% normal rabbit serum were added and the mixture was incubated for 24 h at 4°C. The tubes were then centrifuged at 2000g for 30 min, decanted and the pellet counted for radioactivity on an automatic gamma counter.

Results

Subcellular binding sites for somatostatin

Table 1 indicates considerable somatostatin-binding activity in the various pellets of female rat

Table 1. Distribution of specific binding of ^{125}I -labelled [Tyr^1]somatostatin in subcellular fractions of rat tissues

Particulate fractions were obtained from a number of tissues from female rats as described in the Materials and Methods section: 0.1 ml of each fraction (containing 150 μg of protein in 25 mM-Tris/HCl buffer, pH 7.6) was incubated in glass tubes for 2 h with ^{125}I -labelled [Tyr^1]somatostatin (approx. 25000 c.p.m.) in a total volume of 0.5 ml. The buffer for all dilutions, except for particulate fractions and additions, consisted of 50 mM-Tris/HCl buffer, 1.3 mM- CaCl_2 and 0.5% bovine serum albumin, pH 8.0. All reagents and reaction tubes were kept in ice before and during assay. At the end of the assay period, cold (4°C) ethanol was added to a final concentration of 85% (v/v), and reaction tubes were centrifuged at 2000g for 20 min at 4°C . The radioactivity in the pellets was counted in an automatic gamma counter. Results are shown as means $\pm$ S.E.M. for the number of experiments in parentheses.

Tissues	Specific binding (%)		
	900g pellet	15000g pellet	100000g pellet
Liver (6)	25.7 $\pm$ 1.0	25.8 $\pm$ 0.6	28.8 $\pm$ 0.4
Submandibular gland (4)	18.1 $\pm$ 0.5	14.2 $\pm$ 0.8	12.9 $\pm$ 1.4
Skeletal muscle (4)	4.0 $\pm$ 0.9	4.0 $\pm$ 1.0	7.2 $\pm$ 0.4
Spleen (4)	5.0 $\pm$ 0.5	4.7 $\pm$ 0.2	5.5 $\pm$ 0.6
Stomach (4)	4.3 $\pm$ 0.7	4.4 $\pm$ 0.8	3.7 $\pm$ 0.7
Heart (4)	9.2 $\pm$ 1.2	4.6 $\pm$ 0.3	2.6 $\pm$ 0.8

Fig. 1. Incubation time and the specific binding of ^{125}I -labelled [Tyr^1]somatostatin to cytosol fractions of rat tissues

For this experiment 50 μg of cytosol protein per tube was used, and procedures for determining specific binding are described in the Materials and Methods section. Values are expressed as percentage of total ^{125}I -labelled [Tyr^1]somatostatin in each tube and are means of triplicate determinations. ●, Anterior pituitary; Δ , liver; $\square$, stomach; ■, brain; ○, heart; $\blacktriangle$, skeletal muscle.

Table 2. Identification of subcellular binding sites for somatostatin

The particulate fraction (150 μg of protein/500 μl) or cytosol fraction (100 μg of protein/500 μl) obtained from female rat liver were incubated separately with ^{125}I -labelled [Tyr^1]somatostatin, with or without somatostatin (10 $\mu\text{g}/\text{ml}$), each in a total volume of 7.5 ml, for 4 h in ice. A portion (0.5 ml) of the reaction mixture was used for the determination of specific binding by using ethanol to precipitate bound somatostatin (EtOH method) as described in the Materials and Methods section (Step I). The remainder of the reaction mixture in both cases was centrifuged at 200000g for 60 min and the radioactivity in both pellets was counted (Step II). To determine the specific binding of the 200000g supernatant 0.5 ml of each supernatant from Step II was treated by the EtOH method or the dextran-coated charcoal method as described in the Materials and Methods section (Step III).

	Method for the separation of bound and free tracer	Specific binding of ^{125}I -labelled [Tyr^1]somatostatin (% of total radioactivity)	
		Particulate fraction	Cytosol fraction
Step I	EtOH method	17.5	22.2
Step II	Ultracentrifuge	3.7	0
Step III	EtOH method	13.9	22.9
	Dextran-coated charcoal method	20.9	19.2

tissues. Table 2 indicates that most somatostatin-binding activity occurs in the 200000g supernatant (cytosol fraction) of rat liver. Most of the binding activity in the 100000g pellet can be detected in the supernatant after resuspension of the pellet (Table 2). Similar results were obtained with rat submandibular-gland subcellular fractions.

Effect of EDTA and Ca^{2+}

Three different 50 mM-Tris/HCl buffers at pH 8.0 containing 25 mM-EDTA, no EDTA, or without EDTA but with 1 mM- CaCl_2 , were used for homogenization and incubation. Rat liver cytosol proteins (100 μg per tube) were incubated in ice for 5 h. With 25 mM-EDTA, the specific binding is $25.8 \pm 2.4\%$ (mean $\pm$ S.E.M., $n = 5$). Without EDTA, it is $15.2 \pm 1.4\%$ ($n = 3$) and with 1 mM- CaCl_2 it is decreased to $4.4 \pm 0.4\%$ ($n = 3$).

Optimum conditions for assay of binding activity

The specific binding was maximum after 12–24 h incubation at 4°C for all tissues (Fig. 1), including human and bovine anterior pituitaries (results not shown). Incubation at 24°C or in ice yielded lower

Fig. 2. Effect of amounts of cytosol protein on the binding of ¹²⁵I-labelled [Tyr¹]somatostatin
Incubation conditions and determinations of specific binding are described in the Materials and Methods section. Results shown are means of triplicate determinations. Symbols are the same as in Fig. 1.

Fig. 3. Effect of pH on binding of ¹²⁵I-labelled [Tyr¹]somatostatin
For this experiment 50 µg of cytosol protein was used, and determination of specific binding is described in the Materials and Methods section. The pH of the incubation buffer (0.2M-Tris/25mM-EDTA) was adjusted with 6M-HCl or 10M-NaOH as required. All pH values are the final pH in the incubation media. Values shown are the mean of triplicate determinations. The symbols are the same as in Fig. 1.

maximal binding than at 4°C. We have used 18h incubation periods at 4°C in routine assays.
At 4°C the specific binding of ¹²⁵I-labelled [Tyr¹]somatostatin was proportional to the cytosol protein concentration from 3 to 50 µg per tube (Fig. 2). The pH optima for specific binding is pH8.0–8.5 in all tissues (Fig. 3). The tissues with somatostatin-binding sites in the cytosol are listed in Table 3.

Effects of various conditions on somatostatin binding

The effect of exposure of cytosol to alkali, acid, ethanol or heat on binding of somatostatin is shown in Table 4.

Table 3. Distribution of specific binding of ¹²⁵I-labelled [Tyr¹]somatostatin in cytosol fractions
Incubation conditions and procedures for determination of specific binding of ¹²⁵I-labelled [Tyr¹]somatostatin were similar to those described in the Materials and Methods section, except that 10 µg of cytosol protein and 24h incubation were used for all tissues. Because of the small size of rat anterior pituitary glands, four pituitaries were used as one sample. Values are means ± S.E.M. for the numbers of animals given in parentheses.

Animals	Tissues	Specific binding (%)
Female rat	Skeletal muscle (4)	35.6 ± 1.4
	Liver (4)	33.3 ± 1.1
	Small intestine (4)	28.9 ± 0.7
	Anterior pituitary (4)	26.7 ± 1.2
	Stomach (4)	26.3 ± 0.3
	Spleen (4)	25.2 ± 0.6
	Brain (4)	24.8 ± 1.1
	Submandibular gland (4)	24.6 ± 2.4
	Uterus (4)	21.2 ± 1.7
	Heart (4)	19.4 ± 1.1
	Kidney (4)	19.2 ± 1.4
	Lung (4)	14.9 ± 1.3
	Peripheral blood cells (2)	2.6 ± 0.3
	Plasma (2)	1.2 ± 0.2
Human	Liver (4)	22.4 ± 3.8
	Anterior pituitary (3)	15.7 ± 1.6
	Plasma (2)	1.2 ± 0.8
Bovine	Anterior pituitary (4)	20.7 ± 1.0

Table 4. Effect of various reagents on somatostatin binding
The effects of wide differences of pH, temperature and of exposure to ethanol on the binding-protein activity were studied as explained in the Materials and Methods section. Results shown are means of triplicate determinations.

Treatment	Specific binding of ¹²⁵ I-labelled [Tyr ¹]somatostatin (% of control)	
	Rat liver	Bovine anterior pituitary
Control	100	100
Acetic acid (0.1 M), 30 min at 24°C	23.1	30.8
NaOH (0.1 M), 30 min at 24°C	16.2	23.3
Ethanol(85%,v/v), 20 min at 4°C	58.5	—
30 min at 56°C	0	9.0
1 min at 100°C	0	0
Freezing and thawing (20 times)	95.0	94.6

Effects of enzymes on somatostatin-binding activity

Table 5 shows the effect of incubation of cytosol fraction containing somatostatin-binding activity with various enzymes. Binding activity is decreased

Table 5. *Effect of enzyme treatment on somatostatin-binding activity*

Cytosol fractions were incubated in the presence of different concentrations of enzymes as explained in the Materials and Methods section. Results shown are means of triplicate determinations.

Enzyme	Concn. ($\mu\text{g/ml}$)	Specific binding of ^{125}I -labelled $[\text{Tyr}^1]\text{somatostatin}$ (% of control)	
		Rat liver cytosol	Bovine anterior pituitary cytosol
Control	—	100	100
Trypsin	50	37.9	27.5
	5	73.9	81.1
RNAase	50	92.2	104.3
	5	106.2	108.6
DNAase I	50	101.9	101.4
	5	96.3	99.6
Phospholipase C	50	94.6	108.6
	5	106.2	99.5

Table 6. *Dissociation of the binding between ^{125}I -labelled $[\text{Tyr}^1]\text{somatostatin}$ and somatostatin-binding protein*

The effect of various reagents on ^{125}I -labelled $[\text{Tyr}^1]\text{somatostatin}$ binding to somatostatin-binding protein was studied by methods discussed in the Materials and Methods section. As adsorption of ^{125}I -labelled $[\text{Tyr}^1]\text{somatostatin}$ by dextran-coated charcoal was decreased by some reagents (80–91% instead of 98%), appropriate correction factors were applied. Results shown are means of triplicate determinations. A, Female rat liver cytosol; B, bovine anterior pituitary cytosol.

Treatment		Dissociation of total binding (%)		
		1.5h	12h	24h
Acetic acid (0.1 M)	A	37.9	25.2	23.5
	B	56.0	34.6	23.8
NaOH (0.1 M)	A	65.9	59.9	54.6
	B	67.9	61.9	46.4
Dithiothreitol (2 mM)	A	59.7	65.6	77.9
	B	56.1	74.9	84.7
Mercaptoethanol (5 mM)	A	53.8	66.6	78.7
	B	61.7	71.5	80.9
CaCl_2 (5 mM)	A	29.5	20.5	16.1
	B	44.1	24.7	16.5
MgCl_2 (5 M)	A	29.5	22.2	15.3
	B	43.1	24.3	18.3
Urea (8 M)	A	47.4	35.0	31.0
	B	47.1	32.1	22.5
Guanidine hydrochloride (7 M)	A	52.7	46.4	46.2
	B	55.6	40.7	31.2
NaCNS (5 M)	A	32.9	22.3	17.6
	B	35.6	22.8	15.9
Control (Tris/EDTA/albumin buffer)	A	34.9	20.9	14.7
	B	43.7	22.0	13.1

Fig. 4. *Distribution of bound ^{125}I -labelled $[\text{Tyr}^1]\text{somatostatin}$ after gel filtration on Sephadex G-100*

Cytosol fractions of rat liver (----), human anterior pituitary (—) or bovine anterior pituitary (····) were incubated with ^{125}I -labelled $[\text{Tyr}^1]\text{somatostatin}$. After incubation, dextran-coated charcoal was added and the mixture centrifuged at 2000g for 10 min. Supernatants were applied to a column (1.2 cm $\times$ 50 cm) of Sephadex G-100. Fractions (2 ml) were collected and the radioactivity of each fraction was measured on an automatic gamma counter. A, Blue Dextran; B, ^{125}I -labelled human somatotropin; C, somatostatin and ^{125}I -labelled $[\text{Tyr}^1]\text{somatostatin}$.

significantly only by trypsin exposure, suggesting that protein is a functionally important part of the binding sites.

Dissociation studies

Excess of unlabelled somatostatin is unable to displace ^{125}I -labelled $[\text{Tyr}^1]\text{somatostatin}$ previously bound to somatostatin-binding protein (results not shown). Dithiothreitol and mercaptoethanol enhance dissociation the most, and their effect increases with prolonged periods of preincubation for the binding between the binding protein and the tracer (Table 6). Urea and guanidine hydrochloride dissociate tracer binding somewhat, but increasing preincubation time decreases their ability to do so. Spontaneous dissociation (control) also decreases with increasing preincubation time.

Comparison of somatostatin-binding protein from various sources

Two distinct peaks of radioactivity were noted after gel filtration on Sephadex G-100 of supernatants obtained as described in the legend to Fig. 4. The largest peak (fractions 12–16) appeared just after the void volume, and the second smaller peak was coincident with somatostatin and ^{125}I -labelled $[\text{Tyr}^1]\text{somatostatin}$. Gel filtration on Sephadex G-100 of cytosol from a variety of rat tissues yielded similar elution volumes for somatostatin-binding activity (Figs. 5a and 5b). The mol.wt. of the binding protein was estimated at 80000 in all cases. After polyacrylamide-gel isoelectric focusing of binding proteins from

Fig. 5. Gel-filtration patterns of somatostatin-binding protein

The same Sephadex G-100 column as that used in Fig. 4 was used. In (a), 5.9 mg of cytosol protein of rat liver (Δ) was applied. Eluents were monitored for protein (----) by their A_{280} . In (b), 1 mg of rat anterior pituitary ($\bullet$), 7.6 mg of stomach ($\square$), 6.3 mg of brain ($\blacksquare$) or 5 mg of heart ($\circ$) cytosol protein was applied. The specific binding of somatostatin and the effect of addition of portions ($50\mu\text{l}$) from each fraction on a radioimmunoassay are indicated. The radioimmunoassay was performed as described in Materials and Methods section, except that 1:1000 dilution of rabbit anti-somatostatin serum and 24 h incubation were used.

these tissues, the major somatostatin-binding peak in all cases was found in gel segments at pH 4.6–4.9 (results not shown).

Specificity of the somatostatin-binding protein

By using rat anterior-pituitary cytosol samples, semi-purified by gel filtration on Sephadex G-100, the specificity of the somatostatin-binding protein was determined (Fig. 6). Significant inhibition of the binding occurred with 300 pg of somatostatin. [Tyr^1]Somatostatin and [D-Trp^8]somatostatin cross-reacted 150 and 40% respectively. [$\text{Ala}^3, \text{Ala}^{14}$]Somatostatin, a linear analogue, was only 10% cross-reactive. Surprisingly, pig glucagon and synthetic corticotropin were 7 and 5% cross-reactive respectively. All other hormones tested were less than 1% as active. Similar specificity was obtained with the semi-purified binding protein from rat liver cytosol.

Effects of the somatostatin-binding protein on the radioimmunoassay of somatostatin

Fig. 5 shows that somatostatin-binding protein decreases binding of ^{125}I -labelled [Tyr^1]somatostatin to anti-somatostatin serum. Fig. 7 shows that as little as $1\mu\text{g}$ of somatostatin-binding protein from various tissues decreases the binding of ^{125}I -labelled [Tyr^1]somatostatin to anti-somatostatin serum.

Discussion

The present study demonstrates that somatostatin binds to macromolecules with mol.wt. 80000, found in the cytosol fraction (200000g supernatant) of various rat, human and bovine tissues (Figs. 4 and 5 and Table 3). As specific binding of somatostatin to plasma and peripheral blood cell cytosol is low, it is unlikely that the specific binding of the cytosol fraction is due to contamination by blood elements. The fact that trypsin, but not RNAase and DNAase, destroyed the somatostatin-binding activity, suggests that this somatostatin-binding factor is protein in nature.

This binding protein is not completely somatostatin-specific, and the relative potency of somatostatin analogues (Fig. 6) is somewhat different from bioassay results (Rivier *et al.*, 1975; Vale *et al.*, 1975, 1976; Ferland *et al.*, 1976).

Dissociation studies (Table 6) revealed that the binding between somatostatin and somatostatin-binding protein may be a two-step event, with the first being reversible and not involving thiol groups. The second step would involve disulphide linkage between somatostatin and the binding protein and would be irreversible. The second reaction is suggested because the longer the preincubation of ^{125}I -labelled [Tyr^1]somatostatin with binding protein, the more effective dithiothreitol and mercaptoethanol become in dissociating the binding, but other dissociating agents become increasingly less effective. Also ^{125}I -labelled [Tyr^1]somatostatin, once bound

Fig. 6. Specificity of binding to somatostatin-binding protein

Semi-purified somatostatin-binding protein (10 μ g of protein/tube) obtained after Sephadex G-100 gel filtration of cytosol was incubated with 125 I-labelled [Tyr¹]somatostatin for 48 h at 4°C with or without the addition of various hormones. Non-specific binding of tracer was determined by adding excess of somatostatin (5 μ g/tube) to the incubation mixture before addition of semi-purified binding protein. This value was subtracted from all samples. Competition of 125 I-labelled [Tyr¹]somatostatin binding (expressed as a percentage of binding in the absence of unlabelled somatostatin) was measured in the presence of increasing amounts of somatostatin (●), [Tyr¹]somatostatin (Δ), [D-Trp⁸]somatostatin (□), [Ala³,Ala¹⁴]somatostatin (▲), pig glucagon (■), or synthetic corticotropin (○). The numbers in parentheses represent the percentage cross-reactivity compared with somatostatin. Other hormones were tested at a single dose (1 μ g/tube) and their range of competitive binding lies within the range indicated as less than 1%. These hormones are listed in the Materials and Methods section.

to the binding protein, is not easily displaced by excess of unlabelled hormone. The first step of the binding may involve a unique tertiary structure because several hormones that have disulphide bonds failed to cross-react. [Ala³,Ala¹⁴]Somatostatin and pig glucagon, which lack cysteine residues, but have a four-amino-acid sequence in common with somatostatin (Thr-Phe-Thr-Ser), show low but parallel cross-reactivity for the somatostatin-binding protein. This may mean they interact in the first step, but cannot proceed to the second step. Secretin also contains the same four-amino-acid sequence and recently we showed that it cross-reacts with the binding protein, with 50 μ units of secretin being

Fig. 7. Effect of somatostatin-binding protein on the binding of 125 I-labelled [Tyr¹]somatostatin to anti-somatostatin serum

Semi-purified somatostatin-binding protein (fraction 14 after Sephadex G-100 gel filtration; Fig. 5) obtained from rat anterior pituitary (●), liver (Δ), stomach (□), brain (■) and heart (○) were used as samples in a radioimmunoassay for somatostatin. The radioimmunoassay was performed as described in the Materials and Methods section. A normal standard curve for the somatostatin radioimmunoassay was indicated by (▼).

equivalent to 10 ng of somatostatin (N. Ogawa & H. G. Friesen, unpublished work).

Whether this somatostatin-binding protein is a true receptor for somatostatin is unknown. There may be a plasma-membrane receptor for somatostatin, because significant binding (approx. 20% of that found on the cytosol) of somatostatin to particulate fractions was still noted after resuspension followed by centrifugation (Table 2). It is also possible that the somatostatin-binding protein is an easily solubilized membrane protein. This would explain why residual binding in the 100000g pellet is so easily solubilized on resuspension (Table 2).

There is evidence that the site of action of somatostatin is distal to cyclic AMP synthesis (Borget et al., 1974; Gerich et al., 1975). Moreover melanotropin (Varga et al., 1976) and prolactin (Posner & Bergeron, 1976) receptors have been identified in the Golgi apparatus. Hence one may postulate an intracellular site of action for somatostatin, especially since there are intracellular receptors for other protein hormones. Therefore perhaps it is reasonable to speculate that the somatostatin-binding protein is important in mediating the action of somatostatin.

Somatostatin-binding protein inhibits binding of ^{125}I -labelled [Tyr^1]somatostatin in a radioimmunoassay (Figs. 5 and 7), presumably by competing with the antisera for the tracer. Similar effects were observed with sheep anti-somatostatin serum. Since the somatostatin-binding protein is fairly stable in the acidic conditions (Table 4) commonly used for extracting somatostatin, estimates of somatostatin in such extracts by radioimmunoassay in some cases will be inaccurate.

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