Vol. 87, No. 3, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 13, 1979 Pages 919-927

SOMATOSTATIN BINDING TO PITUITARY PLASMA MEMBRANES

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Received February 23,1979

SUMMARY:

A method has been developed for the study of somatostatin binding to anterior pituitary plasma membranes. When $5x10^{-9}$M [^{125}I]Tyr^1-somatostatin (SA 18 Ci/mmol) was incubated with isolated pituitary plasma membranes (protein = 100 μg), 13.6% of total radioactivity was bound excluding nonspecific binding. The Scatchard plot could be resolved into two distinct components and analyzed to yield: K_1diss = $3.3x10^{-8}$M and K_2diss = $7.7x10^{-6}$M. This binding was shown to be specific for somatostatin.

INTRODUCTION: As part of a study directed at determining the mechanism of action of somatostatin, a method was developed to evaluate somatostatin binding to various tissues. In the present paper, attention was focused on the binding of labelled somatostatin to plasma membranes isolated from bovine anterior pituitary glands. The measurement of somatostatin binding to tissue employs a similar approach to that which has been described for other polypeptide hormones.(1-3)

MATERIALS AND METHODS: Materials: ^{125}I (>350 mCi/ml, SA∿17 Ci/mg) was purchased from New England Nuclear, Boston, MA. D-Trp^8-somatostatin (SRIF), Ala^8-SRIF, and Tyr^1-SRIF were kindly supplied by Dr. Jean Rivier of the Salk Institute, La Jolla, CA. Cyclic somatostatin and Luteotropic Releasing Factor (LRF) were obtained from Bachem, Inc., Torrance, CA. Thyrotropin Releasing Hormone (TRH) was procured from the Sigma Chemical Company, St. Louis, MO. Bovine pituitaries were generously supplied by Litvak Meat Packing Company, Denver, CO. The measurement of 5'nucleotidase was performed utilizing reagents from Sigma Chemical Company.

Methods: Isolation and iodination procedures: Tyr^1-somatostatin was labelled with ^{125}I according to the method of Harris and associates.(4) [^{125}I]Tyr^1-SRIF was eluted from a Sephadex G-25 (fine) column (1.5 cm x 60 cm) with 0.1M acetic acid containing 0.1% gelatin. The specific activity of the labelled product was ∿18 Ci/mmole.

Anterior pituitary plasma membranes were isolated according to the method of Poirier and co-workers utilizing differential centrifugation and sucrose gradient techniques.(5) The purity of the plasma membrane fraction is discussed elsewhere.(5) In the studies presently being reported, the purity of this fraction was ascertained by measurement of 5'nucleotidase activity as well as periodic electron microscopic examination.(6) A ten-fold enhancement of 5'nucleotidase activity (Units/mg protein) was routinely achieved when plasma membrane fractions were compared to homogenates. Electron micrographs confirmed that a relatively pure unit membrane fraction was being examined.

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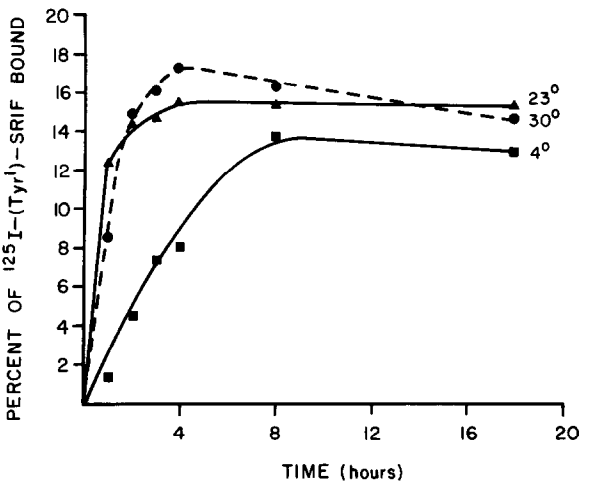

Fig. 1. Effects of time and temperature on binding of [^{125}I]somatostatin to bovine anterior pituitary plasma membranes. Plasma membranes (100 µg protein) were incubated with $5x10^{-9}$M (8 ng/ml) [^{125}I]SRIF at the designated temperatures for the indicated time points. Data are corrected for nonspecific binding.

Incubation for radioactive binding study: Isolated plasma membranes were suspended in 50 mM Tris buffer, pH 7.4 to yield a protein concentration of 1 mg/ml. A 100 µl aliquot of this suspension (100 µg) was added to an incubation media consisting of 400 µl buffer (50 mM Tris, pH 8.0; 25 mM EDTA; 0.5% BSA) and 10 µl of label containing 4 nanograms [^{125}I]SRIF (100,000 cpm). In the studies directed at determining displacement of labelled SRIF, 100 µl of buffer containing unlabelled SRIF was added to yield final concentrations of 0, .01, 0.1, 0.5, 1.0, 2.5, 5.0, 10 and 20 µg/ml. All samples were prepared in triplicate. The samples containing 0 and 20 µg/ml unlabelled SRIF represent "maximum binding" and "nonspecific binding" respectively. Three "total count" samples free of plasma membranes and blanks with no unlabelled SRIF or plasma membranes were included in each incubation. All preparatory steps were carried out at 2°C. The samples were incubated at 4°C for 18 hours. Studies were also directed at defining the optimal pH for the binding reaction as well as determining the effect of time and temperature on somatostatin binding. In all studies the binding reaction (excluding total count tubes) was terminated with the addition of 3.5 ml ice-cold absolute ethanol. The samples were then allowed to stand at 2°C for a period of 10 minutes and subsequently centrifuged at 1700 x g for 30 minutes at 4°C. The supernatant was aspirated and discarded. The pellets were then washed with 2 ml ice-cold 85% ethanol, centrifuged for 10 minutes at 4°C and the supernatant removed. Pellet radioactivity was determined in a Searle Analytic 1185 automatic gamma counting system.

RESULTS: Figure 1 shows the percent binding of labelled somatostatin as a function of time and temperature. When pituitary plasma membranes were incubated with labelled somatostatin at a temperature of 30°C, there was a prompt increase in the amount of [^{125}I]somatostatin bound with binding reaching a maximum at approximately 4 hours. Thereafter, there was a slight decrease in the percent of radioactivity

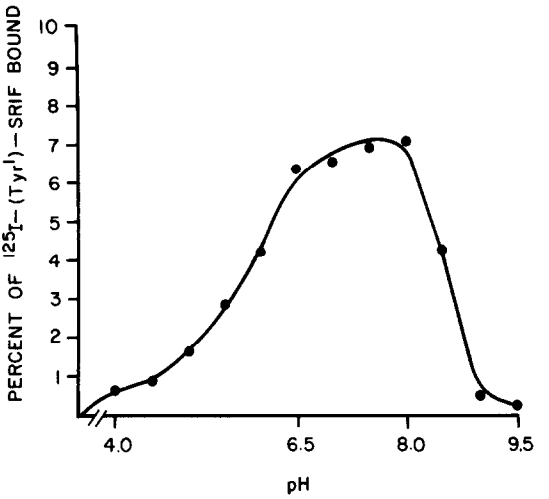

Fig. 2. Effect of pH on [^{125}I]somatostatin binding to anterior pituitary plasma membranes. Plasma membranes (100 µg protein) were incubated with $5x10^{-9}$M (8 ng/ml) [^{125}I]SRIF for 18 hrs at 4°C. Data are corrected for nonspecific binding.

bound to tissue suggesting either the dissociation of somatostatin from the binding sites or deterioration of the binding sites and/or somatostatin. At 23°C the somatostatin binding was slightly slower than that observed at 30°C, but maximum somatostatin binding was still achieved at 4 hours. The uptake of [^{125}I]somatostatin by pituitary plasma membranes was slower at 4°C. The maximum binding at 4°C was reached after 8 hours. Upon having reached the maximum, the percent of labelled binding appeared to remain constant. Maximum binding of labelled somatostatin amounted to 14% of the total radioactivity available, excluding nonspecific binding which was 18%. There did not appear to be any appreciable change in the magnitude of nonspecific binding at the various temperatures studied or with time of incubation. As previously indicated, nonspecific binding is defined as that amount of radioactivity present when the unlabelled somatostatin concentration was equal to 20 µg/ml (tissue protein concentration was 164 µg/ml). This level of SRIF is well in excess of that amount of unlabelled somatostatin needed to achieve maximal displacement of labelled SRIF. In this system, there was no significant difference between the magnitude of the nonspecific binding and that observed in blank samples containing no tissue with or without excess unlabelled SRIF.

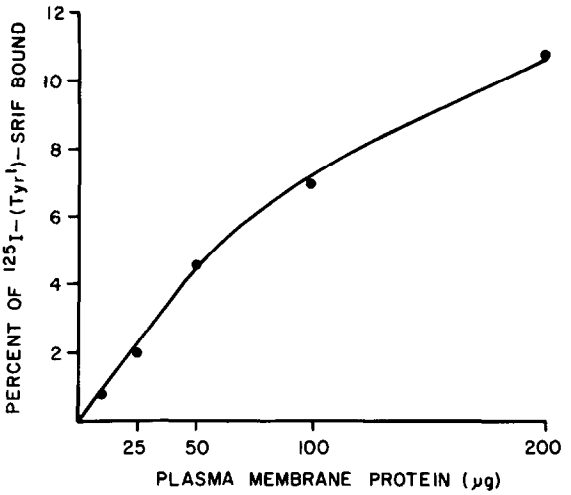

Fig. 3. Effect of plasma membrane protein concentration on [^{125}I]somatostatin binding. Incubations are performed in the presence of 5×10^{-9}M [^{125}I]SRIF for 2 hours at 30°C. Data are corrected for nonspecific binding.

The pH optimum for the binding of somatostatin to pituitary plasma membranes exhibited a relatively broad peak ranging from pH 6.5 to pH 8.0 (Fig. 2). Outside of these pH values, binding appeared to diminish markedly.

Subsequently, studies were undertaken to determine the effect of tissue protein concentration on the binding of unlabelled somatostatin. With the [^{125}]somatostatin concentration maintained at the constant maximal level, there was a good correlation between the percent of label bound and the amount of tissue protein present (r=0.89) (Fig. 3). This correlation was maintained when the percent binding of [^{125}I]somatostatin was expressed per unit of 5'nucleotidase activity, this enzyme recognized to be a relatively good marker for unit membranes (r=0.88). In the plasma membrane fraction, 5'nucleotidase activity was 466 Units/mg plasma membrane protein, with one unit being defined as the micrograms inorganic phosphate generated/mg protein/hr at 37°C.

As the concentration of labelled hormone was increased with a fixed population of receptors (membrane protein held constant), specific somatostatin binding tended to reach a maximum with increasing somatostatin concentration (Fig. 4). This

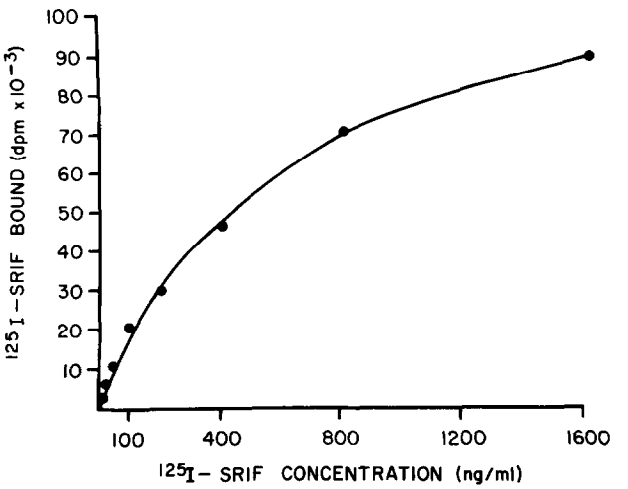

Fig. 4. Effect of increasing [^{125}I]somatostatin concentrations on binding to bovine pituitary plasma membranes (100 µg protein). Data are corrected for nonspecific binding.

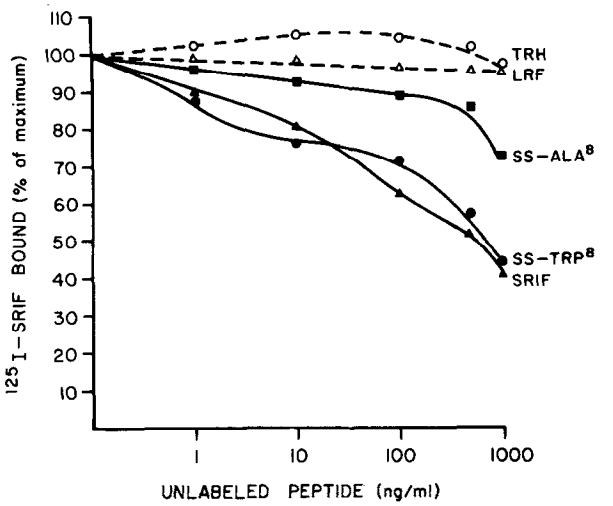

Fig. 5. Inhibiting effect of unlabelled SRIF (▲—▲), Trp^8-SRIF (●—●), Ala^8-SRIF (■—■), LRF (Δ-Δ), and TRH (O—O) on [^{125}I]somatostatin binding to pituitary plasma membranes (100 µg protein). Incubations were carried out for 18 hrs at 4°C in the presence of 5x10^{-9}M [^{125}I]SRIF. Unlabelled peptide concentrations are given on the horizontal axis. Radioactivity which remains bound in the presence of 20 µg/ml SRIF is considered non-specific and has been subtracted from all data points.

curvilinear result suggests that the somatostatin receptor population may be saturated at very high concentrations of labelled hormone.

Relative to the specificity of somatostatin binding, there was no displacement of labelled SRIF by two low molecular weight hypothalamic polypeptides, LRF and TRH

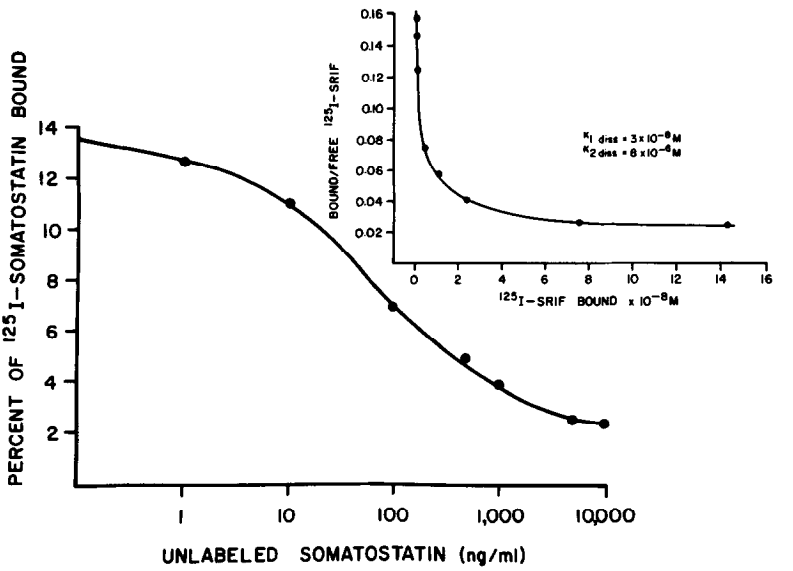

Fig. 6. Inhibiting effect of unlabelled somatostatin on [^{125}I]somatostatin binding to pituitary plasma membranes (100 µg protein). Incubations were carried out at 4°C for 18 hrs in the presence of $5x10^{-9}$M [^{125}I]SRIF. Radioactivity which remains bound in the presence of 20 µg/ml unlabelled somatostatin is considered nonspecific and has been subtracted from all data points.

(Inset): Shows the data when submitted to Scatchard Analysis. The data suggest two orders of binding sites of different affinity, that is a high affinity, low capacity site (Kaff = $3.1x10^{7}M^{-1}$) and a lower affinity, high capacity site (Kaff = $1.3x10^{5}M^{-1}$). In this system, the reciprocal of the Kaff is the Kdiss which is equal to the somatostatin concentration when 1/2 of the receptor sites are bound. Thus, the SRIF concentrations necessary to half-saturate the high and low affinity sites are approximately 53 ng/ml and 13 µg/ml respectively.

(Fig. 5). The specificity of somatostatin for its binding site was examined further employing two synthetic analogs, alanine^{8}-somatostatin and tryptophan^{8}-somatostatin. (7,8) Figure 5 illustrates that there was no displacement with increasing concentrations of the alanine substituted derivative of somatostatin. The tryptophan substituted derivative did show displacement of labelled somatostatin from the binding site comparable to that observed with native hormone.

Figure 6 demonstrates the displacement of [^{125}I]somatostatin by increasing concentrations of unlabelled somatostatin. The inset shows the Scatchard plot obtained from these data. (9) This Scatchard plot can be resolved into two components, K_1diss = $3.3x10^{-8}$M and K_2diss = $7.7x10^{-6}$M (Kdiss = $\frac{1}{\text{Kaff}}$). The affinity

constant of the steep component in the Scatchard plot is high, consistent with the low levels of circulating hormone that produce effects both in vivo and in vitro.

DISCUSSION: Somatostatin appears to bind to pituitary plasma membranes with kinetics which are similar to the binding of polypeptide hormones in other tissue systems. As expected, the velocity of the binding reaction was temperature dependent. To study the characteristics and specificity of the binding reaction, a temperature of 4°C was selected for incubation of the tissue and hormone, i.e., to minimize the possibility of degradation of somatostatin and/or its binding site. Maximum hormone binding was 14% of the total radioactivity available excluding nonspecific binding. When unlabelled SRIF was added, 0.10 μg/ml produced 50% displacement of labelled SRIF. This value is consistent with previously reported SRIF concentrations known to inhibit pituitary hormone release.(10,11)

Somatostatin binding fulfills the requirement of demonstrating saturatability. Increasing the number of binding sites (as measured by increasing tissue protein) results in a rise in somatostatin binding. This binding correlates well with tissue protein concentration as well as with 5'nucleotidase activity. These data suggest that the tissue under study represents a relatively pure fraction of plasma membranes.

The binding site thus far appears to be relatively specific for somatostatin. There was no displacement of labelled somatostatin by LRF, TRH or Ala^{8}-SRIF, a somatostatin analogue which does not possess biologic activity. D-Trp^{8}-somatostatin which in certain tissue systems has enhanced biologic activity showed displacement kinetics similar to the native hormone.

In the present report, the Scatchard plot could be resolved into two dissociation constants as previously noted. Schonbrunn and Tashjian have described somatostatin binding in a tissue culture preparation of $GH_4(C_1)$ cells, a clonal stain of rat pituitary tumor cells which synthesize and secrete prolactin and growth hormone.(12) In their system, they found only one class of high affinity binding sites. The difference between the two studies may be that Schonbrunn and Tashjian were studying a relatively homogenous tissue. Alternatively, they employed 10^{-7}M somatostatin to establish the level of specific and nonspecific binding. In the present

report, 10^{-7}M somatostatin would have resulted in only 50% displacement of the labelled somatostatin. If this level of somatostatin had been utilized in the present study to define specific binding, a similar conclusion would have been drawn, that only one class of binding sites exists.

While certain tissue systems have demonstrated linear binding of polypeptide hormones, in a number of circumstances the binding of various polypeptide hormones has been resolved into two components as in the present study. The significance of high affinity as opposed to low affinity binding sites still remains an issue for discussion. The concept has been advanced that perhaps this type of binding curve represents negative cooperativity, that only one class of binding sites may be present.(13)

It is noteworthy that the magnitude of nonspecific binding observed with the addition of 20 µg/ml SRIF to plasma membranes is the same as that seen in blank samples containing no tissue with or without excess unlabelled SRIF. The explanation of the phenomenon of nonspecific polypeptide hormone binding has been complex and unclear, even though the magnitude of such binding is substantial. If one can extrapolate from the current data employing alcohol precipitation to separate bound and free hormone, it may be that nonspecific binding is not a function of the tissue per se

In previous studies, it has been demonstrated that somatostatin appears to effect secretory processes within specific tissues as well as inhibiting cyclic AMP dependent protein kinase activity.(6,14) Studies are in progress to determine whether there is a difference in somatostatin binding sites in those tissues which are somatostatin sensitive as opposed to those in which somatostatin appears to exert no physiologic effect. Thus far, somatostatin binding activity can be demonstrated in those tissues in which somatostatin inhibits cyclic AMP stimulated protein kinase. Efforts are in progress to correlate the degree of somatostatin binding with inhibition of hormone release and cyclic AMP dependent protein kinase activity.

ACKNOWLEDGMENTS: The authors are indebted to Ms. Dorothy Toothaker for her technical assistance. We wish to express our appreciation to our secretary, Ms. Evelyn Fitzgerald.

This research was supported by the Medical Research Service of the Veterans Administration Medical Center (MRIS#8227), Denver, Colorado, the Diabetes Research Foundation of Denver, Colorado and the Upjohn Company, Kalamazoo, Michigan.

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