Properties of the Interaction between Bovine Thyrotropin and Bovine Thyroid Plasma Membranes*

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Studies have been conducted to characterize further the interaction between 125I-labeled bovine thyrotropin (TSH) and bovine thyroid plasma membranes. Sequential subcellular fractionation of thyroid homogenates yielded preparations of progressively greater specific binding activity, highest activity being found in fractions previously shown to contain predominately plasma membranes (Amir, S. M., Carraway, T. F., Kohn, L. D., and Winand, R. J. (1973) J. Biol. Chem. 248, 4092-4100). Although binding of 125I-TSH by plasma membranes was greatest at pH 6.0, studies were conducted at pH 7.45 as well as pH 6.0, and results obtained differed quantitatively, but not qualitatively.

Binding was maximal at 0°, 15°, and 22° and steady state values remained unchanged for at least 22 hours. At 37°, binding was decreased by 40% at 1 hour; the loss was even greater (65%) at 50°. A similar loss of binding was evident when membranes were preincubated without TSH at 37° or higher and were then incubated with 125I-TSH at 0°. Lineweaver-Burk analysis indicated that preincubation resulted in loss of receptor sites without change in affinity of residual receptors. Addition of Ca2+ (1 to 10 mM) to the preincubation medium prevented the effect of preincubation at 37° by preserving the number of receptor sites without altering their affinity. Under similar conditions, Na+ and K+ were without protective effect.

Membranes bound 45Ca2+ in a specific and saturable manner. Scatchard plots indicated a dissociation constant (Kd) of 9 × 10^-8 M and a capacity (n) of 54 nmol/mg of membrane protein. 45Ca2+ was also displaced from membranes by Mg2+ and Mn2+. Ca2+ had a biphasic effect on binding; low concentrations (1 to 10 μM) added to the incubation mixture stimulated binding, while higher concentrations (0.1 mM) caused inhibition. Mg2+ and Mn2+, at comparable concentrations, were also inhibitory. Na+ and K+ less so. In the case of Ca2+, both the stimulatory and inhibitory concentrations were lower than those required to achieve saturation of Ca2+-binding sites.

Proteolytic enzymes (trypsin, a-chymotrypsin, and pronase) sharply reduced binding of 125I-TSH, owing to a decrease in receptor sites. Phospholipases A and C enhanced binding of TSH, while neuraminidase and β-galactosidase were without measurable effect.

It is generally agreed that binding to receptors in the plasma membrane of target tissues is the initial step in the action of polypeptide hormones, including TSH* (1-5). What is uncertain, however, is the extent to which alterations in binding interactions at the plasma membrane level can account for variations in thyroid function and metabolism under a variety of physiological and pharmacological stimuli. Preparatory to studies of this type, and in an effort to gain additional information concerning the specific binding of TSH to its receptor in the thyroid gland, the present studies, which employed bovine TSH and bovine thyroid plasma membranes, were undertaken. A portion of this work has been described in abstract form (6).

EXPERIMENTAL PROCEDURE

Materials

Bovine Thyroid Glands—Initial experiments were performed with plasma membranes prepared from bovine thyroid glands obtained in the frozen state from the ERCO Co., San Mateo, Calif. (6, 7). Subsequently, experiments were conducted with bovine plasma membranes prepared from freshly killed animals. The experiments reported herein were conducted in the latter preparations.

Glycoprotein Hormones—Small quantities of highly purified bovine TSH were obtained as a gift from Dr. J. G. Pierce, University of California at Los Angeles. For most experiments, bovine TSH was purified from a standard reference preparation supplied by the Hormone Distribution Office, National Institutes of Health, Bethesda, Md. Unlabeled TSH, used for the measurement of nonspecific binding

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to the thyroid membranes, was a commercial preparation (Thytopar) purchased from the Armour Pharmaceutical Co.

**Methods**

**Purification of TSH**—Partially purified bovine TSH (2.5 i.u./mg, and less than 0.1%) was further purified by sequential column chromatography using CM52, DE52, and Sephadex G-100 resins, as previously described (8). As evaluated in the McKenzie assay system (9), freshly purified preparations of bovine TSH contained 10 to 15 i.u./mg. Purified preparations were used either fresh or were lyophilized and stored until needed at -60°C for periods of up to 2 months.

**Iodination of TSH**—Purified TSH, prepared either in our laboratory or supplied by Dr. Pierce, was radioiodinated by means of a technique, which employs stoichiometric amounts of chloramine T and no reductant (7), to a specific activity of 75 to 150 µCi/µg (1 to 2 atoms of iodine/molecule of TSH). Aliquots of TSH were either used directly or were stored frozen at -60°C. No significant loss in binding activity was noted for periods of up to 4 weeks, and binding of the two types of TSH preparations used for radioiodination did not differ appreciably.

**Preparation of Thyroid Plasma Membranes**—The method described by Wolff and Jones (10) was employed to obtain a membrane-rich pellet from homogenates of thyroid glands obtained from freshly killed cattle. At this stage, the method was altered to incorporate the system of discontinuous sucrose gradient centrifugation employed earlier (8). Briefly, the pellet was suspended in 1 mM NaHCO₃ and was applied to a discontinuous sucrose gradient consisting of 45% (17 ml), 40% (17 ml), and 30% (17 ml) of sucrose (density 1.20, 1.18, and 1.13, respectively) in 20 mM Tris-Cl, pH 7.45. Centrifugation was carried out in a Beckman model L5-65 and SW 25.2 rotor at 22,000 rpm for 2 hours. Material from the 30 to 40% and 40 to 45% interfaces was collected, diluted 10-fold with 20 mM Tris-Cl, pH 7.45, and sedimented by centrifugation. The resulting membrane preparations were either used fresh or were divided into small aliquots and were stored in liquid nitrogen for future use. Immediately prior to use, membranes were thawed at 4°C and diluted with appropriate buffer. Clumping was avoided by gently dispersing membranes with a Pasteur pipette. Membranes prepared in this manner were shown to retain responsiveness of the adenylate cyclase system to both TSH and F.

**Binding Assay**—In the standard assay, aliquots of the membrane suspension, usually containing 60 µg of membrane protein per ml, were incubated in Beckman microtubes with 40 to 400 pg/ml of iodinated TSH (1.4 x 10⁻¹⁰ to 1 x 10⁻⁸ M) in a total volume of 300 µl of 20 mM Tris-Cl buffer, pH 7.45, containing 0.5% bovine serum albumin, to minimize adsorption of ¹²⁵I-TSH to glassware.² In experiments conducted at pH 6.0, 20 mM phosphate buffer in 0.5% albumin was used. Incubation was carried out at 0°C for 2.5 hours unless otherwise stated. The samples were then rapidly transferred to a Beckman No. 152 microfuge and centrifuged for 5 min in a cold room. The supernatant was discarded, the upper ends of the tubes sliced off with a razor, and tips of the tubes containing the radioactive pellet were counted in a well-type scintillation counter. Nonspecific binding of ¹²⁵I-TSH was measured by adding to the incubation mixture a large excess of unlabeled TSH (1 i.u.). Specific binding of ¹²⁵I-TSH was calculated as the difference between total and nonspecific binding.

**Treatment of Membranes with Enzymes**—Plasma membranes were incubated with various enzymes in appropriate buffer systems (see "Results") at 37°C for 15 to 20 min. Controls were preincubated under the same conditions, but without enzymes. Thereafter, reactions were stopped, the mixture was centrifuged at 3,000 x g for 20 min, and the resulting supernatant discarded. The pellet was resuspended in either Tris-Cl, pH 7.45, or phosphate buffer, pH 6.0, washed, and resedimented. The pellet was once again suspended in buffer and the binding assay was performed in an aliquot of the suspension, as described above.

**Binding of Calcium**—Bovine thyroid membranes (78 µg of protein) were suspended in 20 mM Tris-Cl with 0.5% albumin, pH 7.45. ¹⁰⁹CaCl₂ (20 ng, 0.05 µCi) and varying concentrations of ⁴⁰CaCl₂ were added and the mixture (650 µl) was allowed to incubate at 5°C for 20 hours. Nonspecific binding of ⁴⁰Ca⁺ was assessed in the presence of ⁴⁰Ca⁺ at a concentration of 300 µg/ml and was subtracted from total binding to obtain values for specific binding of ⁴⁰Ca⁺. Bound and free Ca⁺ were separated by centrifugation of the reaction mixture at 2,500 x g for 10 min. The supernatant was removed and the pellet resuspended in water, without prior washing. The radioactivity was then measured in the presence of Aquasol (New England Nuclear), using a Packard scintillation counter.

**Protein Concentration**—Measurements of protein concentration were performed by the method of Lowry et al. (11), using crystalline bovine serum albumin as the standard. Membrane protein was determined following solubilization of appropriate aliquots in 1 N NaOH for 2 to 3 min in a boiling water bath.

**Results**

**Localization of ¹²⁵I-TSH Binding** (Table I)—Thyroid glands from freshly killed beef were treated as described for the preparation of plasma membranes. Aliquots of the fractions obtained at various steps of the preparative process, each containing the same amount of protein, were incubated with ¹²⁵I-TSH (9.3 x 10⁻¹⁴ M) as in the standard binding assay, and specific binding by each was assessed. When expressed in relation to protein concentration, binding of ¹²⁵I-TSH increased only slightly until the 17,300 x g pellet was obtained, at which point a sharp increase was noted. When this pellet was resuspended and centrifuged at 4,900 x g, the resulting pellet showed further increase in binding. Continuous sucrose density gradient centrifugation of the resuspended 4,300 x g pellet yielded fractions at the 30 to 40% and 40 to 45% interface that showed further enhancement of binding, indicating an approximately 13-fold increase over that found in the original homogenate. Previous studies have demonstrated that the fraction isolated at the 30 to 40% interface is highly enriched in membrane-bound marker enzymes (8). Since

| Table I | Distribution of ¹²⁵I-TSH binding activity in subcellular fractions of bovine thyroid |
|---|---|---|
| Fractions | ¹²⁵I-TSH bound | Relative purification |
| Subcellular | fmol/mg |  |
| Whole homogenate | 0.5 | 1 |
| Cheese cloth filtrate | 0.9 | 1.8 |
| 1,000 x g pellet | 1 | 2 |
| 1,000 x g supernatant | 0.7 | 1.4 |
| 17,300 x g pellet | 3.3 | 6.6 |
| 17,300 x g supernatant | 0.4 | 0.8 |
| 4,300 x g pellet | 5.0 | 10 |
| 4,300 x g supernatant | 9.0 | 4 |

² Binding assay was carried out using standard conditions i.e. in 20 mM Tris-Cl buffer pH 7.45, containing 0.5% bovine serum albumin, for 2.5 hours at 0°C. (For details, see "Results").
fractions isolated at the 30 to 40% and 40 to 45% interfaces demonstrated very similar binding activities, the two were pooled and used for subsequent studies of TSH binding, as other workers have done (12).

Effect of Incubation Temperature—Early experiments were conducted to ascertain the effect of incubation temperature on the binding of ¹²⁵I-TSH in the standard binding assay. When studied after 2.5 hours at pH 7.45, binding of TSH was greatest at 0, 15, and 22°, no difference between these three temperatures being observed. In experiments carried out at 0° in the presence of increasing membrane concentrations, greater than 15% of labeled TSH was bound at pH 7.45, and as much as 70% at pH 6.0. At 37°, however, binding was reduced by 40%, and at 50° by 65%. Similar effects of incubation temperature were observed at pH 6.0.

In view of these findings, the time-course of binding of TSH at 0° and 37° was studied (Fig. 1). At both temperatures, binding of TSH at pH 7.45 was maximum within a very short interval; i.e., within the 10-min period required for processing of samples for counting. The fraction of added TSH bound was similar at the two temperatures. At 0°, the fraction of labeled TSH bound remained essentially unchanged during the subsequent 22 hours. At 37°, in contrast, a progressive decrease in binding occurred up to approximately 2 hours, with no appreciable further decrease up to 5 hours. In other experiments using lower TSH concentrations, the highest binding at 0° was noted after 1.5 to 2 hours. A similar inverse relationship between the concentration of TSH employed and the time required for maximum binding has been noted in other reports (13). Accordingly, all subsequent incubations of membranes with TSH were conducted at 0° and were allowed to proceed for 2.5 hours.

Effect of Salts—The effects of various cations on ¹²⁵I-TSH binding were assessed by enriching the basic incubation medium at pH 7.45 with concentrations of the chloride salts of Na⁺, K⁺, Ca²⁺, Mg²⁺, and Mn²⁺, ranging between 0.1 and 100 mM. As reported previously (6, 8, 14-16), all proved to be inhibitory (Fig. 2). The inhibitory potencies of Ca²⁺, Mg²⁺, and Mn²⁺ were very similar to one another, as were the potencies of Na⁺ and K⁺. A strikingly greater inhibitory effect was observed, however, in the case of the divalent cations, a difference that could not be explained by the differing ionic strengths of the media. Very similar results were obtained in studies conducted at pH 6.0.

In view of the fact that calcium is required for the action of many hormones (17) and is stimulatory to ligand-receptor interactions in certain systems (12), further studies of the effects of calcium were conducted. Preincubation of membranes with EDTA (4.3 mM) for 1 hour at 0° or 37° and pH 7.45, followed by careful washing of the membranes with incubation medium free of EDTA, did not affect the subsequent binding of ¹²⁵I-TSH when assessed under the standard conditions. However, in accord with previous reports (15), direct addition of very low concentrations of calcium (10 to 30 μM) to the standard incubation medium resulted in a significant (approximately 20%) increase in ¹²⁵I-TSH uptake (Fig. 3). Within the same experiments, progressively higher concentrations of calcium were associated with progressive inhibition of binding to values below those found in the absence of calcium.

**FIG. 1.** Effect of incubation temperatures on ¹²⁵I-TSH binding to bovine thyroid membranes. ¹²⁵I-TSH (2.6 x 10⁻¹⁶ M) was incubated with bovine thyroid membranes (60 μg/ml) in 20 mM Tris-Cl buffer, pH 7.45, containing 0.5% bovine serum albumin (Tris/albumin buffer) at 0° and 37°. At the indicated time intervals, aliquots were withdrawn and the extent of binding determined as described under "Methods." Similar results were obtained at pH 6.0.

**FIG. 2.** Effect of cations on the binding of ¹²⁵I-TSH to bovine thyroid membranes. The incubation medium contained ¹²⁵I-TSH (3.5 x 10⁻¹⁵ M), bovine thyroid membranes (60 μg/ml), and increasing concentrations of CaCl₂ (●), MgCl₂ (●), MnCl₂ (●), NaCl (●), KCl (●), or NaCl (●). The incubation was carried out at pH 7.40 and 0°. Similar results were obtained (not shown) when the binding experiment was carried out at pH 6.0.

**FIG. 3.** Effect of adding CaCl₂ to the incubation medium on the binding of ¹²⁵I-TSH to bovine thyroid membranes. Bovine thyroid membranes (620 μg/ml) in Tris-Cl buffer, pH 7.45, containing 0.5% bovine serum albumin (Tris/albumin), were preincubated either at 0° (●) or 37° (●) for 70 min. The membranes (100 μg/ml) were reincubated with ¹²⁵I-TSH (7.4 x 10⁻¹⁴ M) at 0° and pH 7.45, with and without increasing concentrations of CaCl₂. A similar effect of CaCl₂ was seen in experiments conducted at pH 6.0.
In view of this effect of calcium and of evidence that calcium is itself bound to the plasma membranes of other tissues (19-20), studies of the binding of ⁴⁺ to thyroid plasma membranes were conducted (see "Methods").

⁴⁺ was found to bind specifically to the membranes in a saturable manner. In presence of 120 µg/ml of membrane protein, specific and saturable binding of ⁴⁺ was observed, reaching a maximum at a medium concentration of 190 µM (7.6 µg/ml). Nonspecific binding of ⁴⁺ was measured in the presence of excess of cold CaCl₂ and was less than 0.9% of the total. No membrane-bound ⁴⁺ was detected following two washings with 20 mM Tris-Cl, pH 7.45, and 0.5% albumin buffer. Mg²⁺ and Mn²⁺ displaced ⁴⁺, although somewhat less efficiently than did calcium itself. EDTA, at a concentration of 10 mM, was as effective in inhibiting the binding of ⁴⁺ as the equivalent concentration of calcium. Bovine thyroid membranes have a relatively weak affinity for Ca⁺. From data obtained at pH 7.45, a dissociation constant (Kd) of 9 × 10⁻⁵ M and binding capacity (n) of 54 nmol/mg of membrane protein were calculated from a Scatchard plot (Fig. 4) (21). It is likely that the dissociation constant was underestimated, since 0.5% albumin, which itself is known to bind calcium, was present in the incubation mixture.

Effect of Preincubation—In view of the loss of ¹²³I-TSH binding that was observed when membranes and hormone were incubated together at temperatures of 37° or higher, further studies of the effects of temperature were conducted. Membranes preincubated for 1 hour at various temperatures were collected by centrifugation, resuspended in fresh medium, and the binding of ¹²³I-TSH measured under standard conditions at 0°. Membranes preincubated at 0°, 15°, and 22° bound ¹²³I-TSH as well as membranes that had not been preincubated. Following preincubation at 37°, binding was reduced by more than half, and an even greater loss was noted after preincubation at 45° and 55°. Lineweaver-Burk analysis revealed that preincubation at 37° resulted in a loss of receptor sites without change in affinity (Fig. 5). Although loss of binding following preincubation was always noted in many experiments, its magnitude was variable.

In several experiments, it was noted that if preincubation of membranes was performed in media containing calcium, the inhibitory effect of preincubation was apparently prevented. Accordingly, additional experiments were performed to explore this finding. In these, initial observations were confirmed. As seen in Fig. 6, loss of binding activity occurred during preincubation at 37° and pH 7.45, regardless of whether binding was measured at pH 6.0 or pH 7.45. However, the inhibitory effect of preincubation was greater when binding was measured at the former pH. Preincubation of membranes at 37° did not result in subsequent loss of binding if preincubation media contained low concentrations of calcium (1 mM or less). A similar effect was evident when Ca⁺ concentrations up to 10 mM were employed, and the protective effect of calcium was evident regardless of whether binding was studied at pH 6.0 or pH 7.45. The protective effect of calcium during preincubation was reproduced by comparable concentrations of other divalent cations (Mg²⁺ and Mn²⁺), but not by Na⁺ or K⁺. Lineweaver-Burk plots revealed that the protective effect of calcium during preincubation was a reflection of its ability to prevent loss of receptor sites without change in affinity (Fig. 5).

When preincubation of membranes was carried out at 50°, calcium could not completely prevent the loss of subsequent binding. Addition of calcium to the preincubation medium had no effect on subsequent binding when preincubation was conducted at 0°.
Effects of Enzymes—Effects of preincubating thyroid plasma membranes with a variety of enzymes prior to assessment of $^{125}$I-TSH binding were determined. When studied at a single concentration of TSH (8.4 x 10$^{-12}$ M), the proteolytic enzymes, trypsin, chymotrypsin, and pronase, sharply reduced binding of $^{125}$I-TSH at pH 7.45 (Table II). The effect of trypsin was completely blocked in the presence of soybean trypsin inhibitor. When studied at pH 6.0, similar effects of the several enzymes were seen; but, especially in the case of trypsin and chymotrypsin, the decrease in binding was proportionately less than at pH 7.45.

The effects of trypsin and chymotrypsin were studied at varying concentrations of TSH so as to permit analysis of their effects on binding parameters. Regardless of whether binding was studied at pH 7.45 or pH 6.0, both enzymes produced a decrease in binding capacity associated with an increase in affinity (Figs. 7 and 8). Compared to the effects seen at pH 7.45, the increase in binding affinity was greater and the loss of binding sites smaller at pH 6.0. This would explain the relatively smaller decrease in the percentage uptake of $^{125}$I-TSH induced by the enzymes when studied at the lower pH.

When studied at pH 7.45, binding of $^{125}$I-TSH was increased by preincubation of membranes with phospholipase A and C. Analysis of binding parameters revealed that this was due to an increase in affinity without a significant change in the number of binding sites. When binding was studied at pH 6.0, these effects were present, but less marked. Preincubation with phospholipase D had no effect on binding at pH 7.45.

Regardless of whether binding studies were conducted at pH 7.45 or pH 6.0, preincubation of membranes with β-galactosidase or neuraminidase at their respective pH optima (7.5 and 5.0) had no effect on binding of $^{125}$I-TSH. A similar lack of effect was evident when membranes were preincubated with DNase. As has been noted previously (22), slight loss of binding followed preincubation with RNase, possibly because of slight contamination of the preparation by proteolytic enzymes.

**Table II**

| Enzyme treatment | $^{125}$I-TSH Bound |
|------------------|---------------------|
|                  | pH 7.45 | pH 6.0  |
| None             | 100     | 100     |
| Trypsin*         | 45 ± 0.56 | 83 ± 2.5 |
| Trypsin + inhibitor* | 100 ± 3.1 | 100 ± 2.0 |
| Chymotrypsin*    | 50 ± 1.3 | 83 ± 1.1 |
| Pronase*         | 32 ± 2.7 | 46 ± 6.0 |
| Phospholipase A* | 168 ± 3.5 | 155 ± 1.8 |
| Phospholipase C* | 158 ± 5.5 | 120 ± 4.2 |
| Phospholipase D* | 95 ± 6.0 | 126 ± 1.9 |
| Neuraminidase*   | 82 ± 0.31 | 110 ± 4.7 |
| β-Galactosidase* | 38 ± 0.6 | 97 ± 0.9 |
| Neuraminidase & β-galactosidase* | 98 ± 2.5 | 93 ± 2.4 |
| D*Nase*          | 96 ± 2.5 | 93 ± 2.4 |
| R*Nase*          | 89 ± 6.0 | 75 ± 4.0 |

*The enzyme-substrate ratio was 1:15 for trypsin, chymotrypsin, and pronase. Incubation was at pH 7.5 for 15 min at 37°C. In control experiments, trypsin inhibitor at twice the concentration of trypsin was added prior to the addition of the enzyme.

The enzyme-substrate ratio was 1:200 for phospholipase A and 1:6 for phospholipase C and D. The incubation with phospholipase A and C was at pH 7.5 at 30°C for 10 min. Phospholipase D was incubated at pH 5.6 and 37°C for 15 min. The medium in all cases contained 1.8 mM CaCl$_2$ and the reaction was stopped by addition of EDTA (2.8 mM).

The medium contained an enzyme-substrate ratio of 1:18 for neuraminidase and β-Galactosidase. Incubation was at pH 5.0 for neuraminidase and 7.5 for β-galactosidase and was continued for 15 min at 37°C.

*Incubation was first performed with neuraminidase, the enzyme removed and the membrane reincubated with β-galactosidase as noted in Footnote c.

*Incubation of membranes with DNase and RNase was carried out at an enzyme-substrate ratio of 1:9 at pH 7.5 and 37°C for 15 min. In case of DNase, the medium also contained 3 mM MgCl$_2$.

**DISCUSSION**

The present studies have served to define certain characteristics of the TSH receptor in bovine thyroid plasma membranes and of its interaction with bovine TSH. In addition to confirming the findings of previous workers in the same or similar systems, a variety of hitherto undisclosed properties have been uncovered.

We first examined the advantages of studying TSH binding in fractions highly enriched in plasma membranes, as most previous workers have done, rather than in cruder fractions. As would be expected if specific binding was localized to the plasma membranes, preparative procedures yielding fractions known to be progressively enriched in membrane marker enzymes yielded a progressive increase in the binding activity per unit weight of protein. The specific binding of TSH seen in fractions containing primarily other cellular organelles probably represents contamination by plasma membranes. The...
fractions ultimately employed for study, which have been shown by electron microscopy to be comprised predominantly of plasma membranes (8), were also demonstrated to have both TSH- and fluoride-stimulated adenylate cyclase activity, indicating that the receptor-cyclase complex was relatively intact.

In accord with most previous studies of bovine thyroid membranes (6, 15, 16), but not all (23), binding of TSH was found to be optimal at acid pH (6.0), rather than the physiological pH of 7.45. In order to evaluate binding at both optimum and physiological pH, we have conducted all of the remaining studies of the binding interaction at both pH 6.0 and pH 7.45. In general, all phenomena found at one pH were qualitatively reproducible at the other, though quantitative differences were seen.

As in previous studies of bovine thyroid membranes under conditions similar to our own, binding of \(^{125}\text{I}\)-TSH was greater when incubations were conducted at 0° than at 37° (8, 15). Lesser binding at the higher temperature was apparently due to progressive alteration of the membrane with time, as shown in two types of experiments. In the first, the fraction of \(^{125}\text{I}\)-TSH bound by membranes decreased progressively with time in incubations carried out at 37°, but not at 0°. In the second, preincubation at 37° prior to addition of TSH was shown to result in a loss of binding when this was subsequently studied at 0°. Such loss was shown by Lineweaver-Burk analysis to be due to a loss of binding sites at 37°, without change in receptor affinity. Tate and co-workers (19) have also reported a loss in TSH binding following preincubation of membranes at 37°, but in contradistinction to the results that they report, we found no evidence that this loss is reversed by reincubating the membranes at 0°. Indeed, in our experiments, binding was always assessed following preincubation by incubating membranes with \(^{125}\text{I}\)-TSH for 2.5 hours at 0°, and the loss induced by preincubation was consistently seen. Additional evidence that the loss of binding during incubation at 37° was due to a change in the receptor, rather than in the TSH, was obtained from rebinding experiments. In these, \(^{125}\text{I}\)-TSH contained in the media, following incubation with thyroid membranes at 37° for 1 hour, was shown to bind to fresh membranes to the same extent as the \(^{125}\text{I}\)-TSH preparation that had not been preincubated.

Among the multiple effects of calcium that we have noted is the ability of low concentrations (0.1 to 1.0 mM) to diminish or completely prevent the loss of binding sites that occurred during preincubation. This effect was shared with Mg\(^{2+}\) and Mn\(^{2+}\), but not with the monovalent cations Na\(^+\) or K\(^+\). Moreover, to exert its protective effect, calcium had to be present during the preincubation, since addition of Ca\(^{2+}\) during reincubation of membranes at 0° did not restore binding of TSH by membranes preincubated at 37° to control values.

No information is available concerning the mechanism of this loss of binding sites, but it could arise from autodigestion within the membranes, aggregation, or conformational changes that occlude potential binding sites. Neither is it clear how calcium exerts its protective effects. Since, as is discussed below, calcium has been found to bind to thyroid plasma membranes in a reversible and saturable manner, it is possible that calcium bound at or near the TSH receptor may protect it from one or more of the inhibitory factors mentioned above.

A second phenomenon related to calcium was the demonstration that Ca\(^{2+}\) undergoes saturable binding to thyroid plasma membranes, in apparent similarity to the binding of calcium to plasma membranes of the rat hepatocyte (18) and salivary gland (19), and to synaptic plasma membranes (20). Binding of Ca\(^{2+}\) was of a lower affinity than that of TSH (approximately 10\(^{-3}\) M\(^{-1}\) versus 10\(^{-6}\) M\(^{-1}\)), but the concentration of ionized calcium in extracellular fluid is of the order of 10\(^{-3}\) M. Moreover, the binding capacity for calcium was far higher than that for TSH (approximately 50 nmol/mg of protein versus 40 pmol/mg of protein). Binding of calcium was not entirely specific, since 4Ca\(^{2+}\) could be displaced nearly as well by the divalent cations, Mg\(^{2+}\) and Mn\(^{2+}\), but not by Na\(^+\) or K\(^+\).

A third group of findings in relation to calcium was the observation that very low concentrations of calcium enhanced binding of \(^{125}\text{I}\)-TSH by thyroid plasma membranes, as also reported by Moore and Wolff (15), while higher concentrations were inhibitory. These effects could not be directly correlated with the binding of calcium, since both stimulatory and inhibitory concentrations of calcium were below those required to saturate the binding sites on the plasma membranes. The nature of the interaction of calcium and TSH at the thyroid plasma membrane or cellular level is uncertain. In vivo effects of TSH and of dibutyl cyclic AMP on glucose oxidation, phospholipid synthesis, and iodine organification are not seen when calcium is absent from the medium (24–26). Involvement of calcium has been indicated in at least two of the effects of TSH on thyroid, namely iodide transport and intracellular glucose metabolism (27). Both in vivo and in vitro, TSH and long acting thyroid stimulator (LATS) have been reported to stimulate calcium uptake by rat thyroid gland, and it has been postulated that the increased uptake may be important for thyroid hormone synthesis (28). Thus, it is entirely evident that TSH and calcium interact in some manner at the level of the thyroid plasma membrane or intracellularly, and the further exploration of this interaction is warranted.

The final group of studies we performed concerned the effects of various enzymes on the binding of \(^{125}\text{I}\)-labeled bovine TSH. In accord with the report of Moore (29), but in contradistinction to the findings of Tate and co-workers (16), who reported that neuraminidase treatment decreased the binding of TSH by solubilized receptors, we found no significant effect of neuraminidase on the subsequent binding over a wide range of concentrations of TSH, at either pH 6.0 or pH 7.45. \(\beta\)-Galactosidase was also without effect, either alone or in combination with neuraminidase. Our findings would indicate that the available sialic acid and galactose residues generally believed to be the constituents of plasma membranes are not essential for binding of TSH.

In contrast, treatment of membranes with either proteolytic enzymes or phospholipases had a pronounced effect on TSH binding. Trypsin, chymotrypsin, and the bacterial protease, pronase, all inhibited binding at both pH values studied, pronase being the most potent in this respect. Similar effects of pronase or trypsin on TSH binding have been noted by others (6, 13, 15). In the case of chymotrypsin and trypsin, Lineweaver-Burk analysis in the present studies revealed that decreased binding was due to a loss of receptor sites, although affinity of the residual receptors appeared to be increased. Whether the latter change represents a true increase in affinity or is merely a reflection of a drastic reduction in the number of residual receptor sites is uncertain (30). Moreover, it is not known whether the proteolytic enzymes destroy the receptors or mainly act to solubilize them, as suggested by the data of Tate and co-workers (13, 10).
A role of membrane phospholipids is suggested by the increase in TSH binding that followed incubation with phospholipases. In confirmation of the findings of Moore and Wolff (15), phospholipase A increased binding of TSH. We have found, in addition, a similar effect of phospholipase C and have obtained data indicating that this is due to an increase in the affinity of the receptor without a change in the number of receptor sites.

It is significant that treatment of thyroid membranes with phospholipase A and C has been found to inhibit stimulation of adenylate cyclase by TSH (31). It appears, therefore, that the integrity of those phospholipids that are perturbed by these enzymes, although not required for hormonal binding, may be necessary for the function of the transducing mechanism and for the maintenance of contact between the regulatory and catalytic subunits of adenylate cyclase enzyme.

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