Thyroid-stimulating Hormone Binding to Beef Thyroid Membranes

RELATION TO ADENYLATE CYCLASE ACTIVITY

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SUMMARY

Binding of [3H]thyroid-stimulating hormone (TSH) and 125I-TSH to beef thyroid membranes was studied under conditions to give optimum binding. Binding was maximal at pH 5.5, was complete within 5 min, and was stable for at least 90 min at 0 and 22°C for low and high TSH concentrations, respectively. At pH 7.5, two binding sites were found with apparent affinity constants of 2.2 to 3.1 × 10^6 M^{-1} and 0.7 to 1.1 × 10^6 M^{-1}. Because of lower specific activity, [3H]TSH was used primarily in the concentration range of the low affinity constant while 125I-TSH was used at concentrations corresponding to both constants. Binding of [3H]TSH and 125I-TSH was equivalent when measured in the same concentration range.

TSH binding was inhibited by divalent cations (Ca^{2+}, Mg^{2+}, Sr^{2+}, Mn^{2+}, and Ba^{2+}) at concentrations above 0.1 mM by reducing the number of binding sites. Monovalent cations inhibited binding at concentrations above 10 to 15 mM. The order of potency for inhibition of binding by monovalent cations was Na^+ > K^+ > Li^+. Inhibition of binding by cations was independent of TSH concentration.

When binding was determined under adenylate cyclase assay conditions, TSH binding was reduced to approximately one-third of the control levels and the higher apparent affinity constant was reduced to 1.6 × 10^6 M^{-1} whereas the second constant was unchanged. These constants coincide roughly with the midpoints of the concentration curve of TSH activation of adenylate cyclase.

TSH was not displaced from the membranes by ITP and GTP at concentrations which enhance cyclase but only at concentrations above 1 mM; the effect of ITP and GTP on binding was independent of medium conditions, TSH concentration or the presence of a regenerating system.

TSH binding studied in the presence of activators and inhibitors of adenylate cyclase permitted classification of the compounds into four groups: (a) agents which affect cyclase and TSH binding in a parallel fashion (Ca^{2+}, Na^+, Li^+, pronase, and cobramine B); (b) agents which enhance cyclase but decrease TSH binding (K^+ and Mg^{2+}); (c) agents which inhibit cyclase but enhance binding (phospholipase A and filipin); (d) agents which alter cyclase but not binding (ITP, GTP, F^-, chlorpromazine, gramicidin S, valinomycin, and cetyl pyridinium chloride).

It is concluded that: (a) TSH binding is relatively stable and major structural alterations are required to affect binding; (b) the effect of activators and inhibitors on TSH binding is independent of TSH concentration and medium conditions; (c) most modulators act in the cyclase system at a stage beyond hormone binding; (d) the response of TSH binding to nucleoside triphosphates indicates that the thyroid membrane system is different from other membrane systems; and (e) the relationship between hormone binding and cyclase activation is complex and direct correlation between binding and activation is not presently possible.

During the stimulation of target tissues by hormones, it is virtually unavoidable that contact and presumably binding of hormones to the plasma membrane is one of the earliest events. In the thyroid gland the question of such an interaction was first approached by indirect means (1) and direct measurement of binding has only recently been successful. Haye and Jacqueemin (2) found saturable binding of labeled TSH to pig thyroid slices that paralleled the stimulating effect on 32P incorporation into lipid. Manley et al. (3) reported specific (i.e. displaceable) binding of 125I-TSH to guinea pig thyroid slices, and Lissitzky et al. (4) obtained similar binding to isolated porcine thyroid cells. Amir et al. (5) found that binding of TSH as a function of concentration paralleled adenylate cyclase stimulation in bovine thyroid membranes.

Bovine and canine thyroid membranes have been shown to have an adenylate cyclase that is responsive to TSH, F^-, prostaglandin E_1 (PGE_1), and sometimes to long-acting thyroid stimulator (6, 7). However, in these systems larger concentrations of TSH were required to obtain cyclase activation than...
would be expected from the published affinity constants (3-5). Similar discrepancies between hormone levels required for cyclase activation in vitro and hormone concentrations that obtain in vivo have been noted for adrenal, renal, and liver tissues (8-11).

It has been possible to manipulate the response of the thyroid membrane adenylate cyclase to TSH by use of a variety of activators (ITP, GTP, K\textsuperscript{+}, etc.) and inhibitors (phenothiazines, ionophores, detergents, Li\textsuperscript{+}, cobramine, etc.) (6, 7, 13). These agents thus offer an alternative approach for the evaluation of the relation between hormone binding and the activation of adenylate cyclase. It was the purpose of the present study to explore these relationships. The results show that no simple relations exist between TSH binding and adenylate cyclase activation.

**EXPERIMENTAL PROCEDURE**

**Materials**—Purified bovine [\(^3\)H]TSH, prepared as described by Winard and Kohn (14), and unlabeled bovine TSH (5 to 10 units per mg) were generously supplied by Dr. Leonard Kohn of this institute. Bovine TSH was iodinated with [\(^125\)I]Iodine-131 by a modification of the lactoperoxidase method of Theorell and Johansson (15). Equimolar amounts (0.2 n mole) of TSH, based on protein determination by the method of Lowry et al. (16) using bovine serum albumin as a standard, and [\(^125\)I]Iodine-131 (100 aci per \(\mu\)l) were added to a vial containing 0.4 M acetate buffer, pH 5.6, and 2 \(\mu\)g of lactoperoxidase (Calbiochem). The reaction was started by adding 0.2 \(\mu\)l of \(H_2O_2\) and was allowed to proceed for approximately 30 s. It was then stopped by adding 100 \(\mu\)l of a solution of 16% sucrose, 1% KCl, and 0.02% sodium azide in 0.1% bovine serum albumin. The mixture was chromatographed on a column (1.5 X 100 cm) of Sephadex G-100. The elution resulted in two symmetrically activity peaks corresponding to iodinated TSH and free [\(^125\)I]Iodine-131. Approximately 40% to 50% iodination was obtained by this method, the specific activity was approximately 1.15 \(\mu\)Ci per pmole. Prostaglandin \(E_1\) was supplied through the kindness of Dr. John Pike of Upjohn Co., Kalamazoo, Michigan, and the prostaglandin antagonists 7 oxo 13 prostaenoic acid and indomethacin were gifts of Dr. J. Fried, University of Chicago and Merck, Sharp and Dohme, respectively. Pure phospholipase A was one of 5 chromatographically distinct phospholipases obtained from Naja nuja venom by repeated chromatography on carboxymethyl cellulose. Its specific activity against egg yolk emulsion was 2.8 pmoles of acid per \(\mu\)g per min. Cobramine B was prepared from \(\alpha\)-vino \(\alpha\) naja venom (17). Pronase (45,000 proteolytic units per g) was purchased from Calbiochem, La Jolla, California. L-Propramycin, filipin, and valinomycin were gifts from Smith, Kline & French, the Upjohn Co., and Lederle Laboratories, respectively. Gramicidin S was a gift of Dr. Mer Willebeek.

**Preparation of Membranes**—Bovine thyroid membranes were prepared as detailed previously (18), except that 0.25 \(\mu\)m sucrose-3 mm Tris HCl (pH 7.5) was used as the suspending solution after the initial homogenization. This was done to eliminate the 1 mm ethylene glycol bis(\(\beta\)-aminoethyl ether)-N,N'-tetraacetic acid and 0.2 mm MgCl\(_2\) which was present in the homogenizing solution. The membranes sedimenting to the 30 to 40% sucrose interface were collected, diluted, centrifuged at 40,000 X g for 20 min, resuspended in 0.25 m sucrose (pH 7.5), and stored over liquid nitrogen until used within 30 to 60 days.

**Analysis of Binding**—Except for the measurement of affinity constants, binding of TSH was routinely carried out under three conditions. (a) Low TSH concentrations (the solution contained 0.7 pmole per ml of [\(^125\)I]TSH, 1.03 to 1.83 pmg per ml of membrane protein (added last), 0.125 m sucrose, 50 mm Hepes (pH 7.5), and 1.25% crystalline albumin in a total volume of 0.1 ml and was incubated at 0 to 2\(^\circ\)C for 15 min. (b) High TSH concentrations (the solution contained ~900 pmole per ml of either [\(^3\)H]TSH or [\(^125\)I]TSH and was incubated in the same media as a but at room temperature). (c) Adenylate cyclase conditions (6, 7). The solution contained 750 pmole per ml of [\(^125\)I]TSH, 0.33 mm cAMP, 2 mm MgCl\(_2\), 25 mm Tris-HCl (pH 7.5), 0.5 mg per ml of phosphocreatine kinase, 6.7 mm creatine phosphate, 0.1% albumin, 1 mm ATP, 0.05 mm ITP, and 0.3 mg per ml of membrane protein in a total volume of 0.12 ml). The membranes were incubated for 10 min at 37\(^\circ\). Assays a and c are referred to in the text as "binding" conditions and c as cyclase conditions. Nondisplaceable TSH was determined for each observation by adding 3.5 nmoles (1 mg per ml) of cold TSH to the incubation mixture. In line with common usage we have called this fraction nonspecific TSH binding. This value was subtracted from the total amount bound to yield specific binding which is reported unless otherwise indicated.

Modulators were added in 5- to 10-\(\mu\)l volumes and controls contained equivalent volumes of the appropriate solvent. All salts were used as the chlorides.

After incubation, the mixture was layered on a discontinuous gradient of 250 \(\mu\)l of 0.3 m sucrose, pH 7.5, with 0.1% albumin on 30 \(\mu\)l of 1.75 m sucrose in a microfuge tube at the incubation temperature. The tubes were centrifuged at 18,000 X g for 10 min at 4\(^\circ\)C in a Sorval SS-34 fixed angle rotor fitted with special adaptors. The stability of the gradient during centrifugation was ascertained by the minimal mixing of [\(^14\)C]glucose, [\(^14\)C]sucreose, or trypan blue added to the top layer. Blank values, in the absence of membranes, of 0.3 to 0.5% of the total radioactivity layered on the gradient were obtained routinely. Similar values were obtained by centrifuging the membranes into a pellet from the incubation solution, aspirating the supernatant, and counting the pellet. The method of Salomon et al. (19) was preferred because it yields lower values for nonspecific binding.

After centrifugation, the tubes were sliced several millimeters above the membranes layered on the 1.75 m sucrose. The sections of the tubes containing the supernatant solution and the membrane were placed in 10 ml of Bray’s solution for [\(^3\)H]TSH counting in a Packard Tri-Carb scintillation counter. The [\(^3\)H]TSH was counted in a Packard gamma counter. About 10,000 cpm of the [\(^3\)H]TSH and 75,000 cpm [\(^125\)I]TSH were present in the incubation mixture. Background counts were 18 to 20 cpm and 40 to 50 cpm, respectively, and samples were counted to less than 3% counting error. The counts in the membrane layer represented bound TSH while counts in the supernatant represented free TSH. Approximately 95% of the radioactivity was recovered in the two fractions. Adenylate cyclase was determined by the method of Salomon et al. (19).

**RESULTS**

Under "binding" conditions at both high and low TSH concentrations, a rapid, concentration-dependent binding of [\(^125\)I]TSH is readily observed. At low TSH concentrations there is a progressive decrease in the specific binding with increasing temperature from 2 to 60\(^\circ\). This decrease is accompanied by a smaller increase in the nonspecific [\(^125\)I]TSH bound (Fig. 1A). The binding of TSH at concentrations (0.9 \(\mu\)m) comparable to those used in adenylate cyclase assays showed little temperature sensitivity up to 37\(^\circ\) and was routinely assayed at room temperature. Since the specific activity of [\(^3\)H]TSH was sufficient to allow binding analyses at these TSH concentrations, a number of experiments with TSH concentrations of this magnitude were carried out with TSH labeled with this isotope (19) when [\(^3\)H]TSH and [\(^125\)I]TSH binding were compared at equivalent concentrations, they behaved identically, i.e., the label did not influence the results. Similar conclusions regarding these two labels have been reached for angiotensin binding (11). Binding at both high and low TSH concentrations was maximal by ~3 min (i.e., the time required for mixing and layering on gradient) and was routinely assessed after a 15-min incubation. Binding at high ([\(^3\)H]TSH) and low ([\(^125\)I]TSH) TSH concentrations was stable at 22 and 2\(^\circ\), respectively, during a 90-min incubation period. The TSH bound at high and low concentration was displaced within 5 min to nonspecific levels by addition of excess (35 \(\mu\)m) unlabeled TSH. However, the fraction of nonspecific TSH bound was a function of hormone concentration. At high concentrations ([\(^3\)H]TSH), the nonspecific fraction was only 0.1 to 0.2% greater than the membrane-free blank (Fig. 1B). On the other hand, the nonspecific TSH binding at low concentrations formed a significant fraction of the total binding (Fig. 1B). The per-
A, effect of temperature on TSH binding to bovine thyroid membranes. The medium contained 0.7 nM TSH, 0.125 M sucrose, 50 mM Hepes (pH 7.5), and 1.25% albumin in a final volume of 0.1 ml. The reaction was started by addition of 1.33 mg per ml of membrane protein and was terminated after 15 min. Results are expressed as picomoles of TSH bound per milligram of membrane protein ± SD for specific (○) and nonspecific (□) TSH binding. B, effect of TSH concentration on the nonspecific TSH binding to bovine thyroid membranes. Incubation conditions as in A. Incubation temperature was 22-24°C and 0-2°C for [3H]-and [125I]-TSH, respectively. The nonspecific binding is expressed as a fraction ± SD of the total [3H]TSH (●) or [125I]-TSH (□) bound by the membranes.

The capacities of these sites were 1.6 and 340 pmol per mg of membrane protein, respectively. A third very low affinity site with an apparent affinity constant of 4.8 x 10^4 M⁻¹ was not further investigated. From the pH curve (Fig. 2) the constants would be expected to be as much as an order of magnitude greater at the optimum pH.

**Effect of Cations**—Since several cations are known to alter adenylate cyclase activity (6, 12), selected monovalent and divalent cations were investigated for their ability to alter binding of labeled TSH. The three monovalent cations all inhibited TSH binding at 50 mM but showed little effect at 5 mM. The order of potencies was Na⁺ > K⁺ > Li⁺. Sodium fluoride (10 mM) did not affect TSH binding at either low or high concentrations of the hormone.

At a concentration of 5 mM all divalent cations tested (Ca²⁺, Mg²⁺, Sr²⁺, Ba²⁺, and Mn²⁺) reduced the binding of labeled TSH to the membranes. There was a small stimulation of TSH binding at 30 μM Ca²⁺, Mg²⁺, and Sr²⁺ (Fig. 4) which was less prominent in several other membrane preparations. Barium gave similar results. All divalent cations tested inhibited TSH binding at concentrations greater than 1 × 10⁻⁵ M. This was true at both high and low TSH concentration (Fig. 4). Since 15 to 20 mM monovalent cation is required to inhibit TSH binding, the inhibition is probably not due to chloride.

Scatchard plots of TSH binding (at 22°C) constructed at three calcium concentrations (Fig. 5), demonstrate that the Ca²⁺ effect on TSH binding resulted from an alteration of the number of binding sites and not of the affinity for TSH.

Since increases in calcium and hydrogen ion concentration are known to cause aggregation of membrane suspensions (20), we investigated the effect of Ca²⁺ and pH on light scattering (absorbance at 700 nm) as a measure of membrane aggregation.
Fig. 3. Scatchard plots of TSH binding to bovine thyroid membranes. A, Scatchard plot of TSH binding at high TSH concentrations. The incubation solution contained 1.19 mg per ml of membrane proteins, 0.125 M sucrose, 50 mM Hepes (pH 7.5), 1.25% albumin, and the appropriate concentration of [125I]TSH. Incubation was performed at 22-24°C for 15 min. The amount of TSH bound (picomoles per mg of membrane protein) is plotted versus the ratio of amount bound to concentration of free hormone (BOUND/FREE). B, Scatchard plot of TSH binding at low TSH concentrations. The incubation conditions are as in A except that the temperature was 2-4°C. The relation between the high and low affinity site is illustrated in the inset on A. C, Scatchard plot of TSH binding under cyclase conditions. The incubation conditions contained 0.3 mg per ml of membrane protein, 0.33 mM cAMP, 2 mM MgCl₂, 1 mM ATP, 0.1% albumin, 0.05 mM ITP, 6.7 mM creatine phosphate, 0.3 mg per ml of creatine phosphokinase, 25 mM Tris-HCl (pH 7.5), and the appropriate TSH concentration. The incubation was performed at 37°C for 10 min and was initiated by adding membranes to the solution. The inset illustrates the effect of TSH concentration on adenylate cyclase activity in bovine thyroid membranes under identical conditions.
brane aggregation strongly inhibit TSH binding. These observations suggest that TSH binding does not show a simple relationship to the state of aggregation of the membranes.

Effect of Nucleoside Triphosphates—Nucleoside triphosphates (ITP, GTP, and dGTP) elicit marked enhancement in the response of adenylyl cyclase of thyroid membranes to TSH and PGE₁ (7). The exact locus of this effect is not known but from inhibition of F⁻-stimulated cyclase and displacement of glucagon from liver membranes (21, 22), it has been suggested that at least one of the sites for nucleoside triphosphate action is at or on an early step in activation. ITP and GTP both inhibited TSH binding, but unlike their effects on adenylyl cyclase, inhibition of binding occurred only at concentrations of ITP and GTP above 0.1 mM (Fig. 7, A and B). There is thus no correlation between the inhibition of binding and enhancement of the adenylyl cyclase. In fact, the inhibition of TSH binding correlates with the inhibition of adenylyl cyclase activity observed at high concentrations of ITP and GTP. At concentrations of 2 mM, dGTP, UTP, CTP, TTP, and ATP all inhibited TSH binding by 20 to 40% (data not shown). Moreover, the inhibition of TSH binding produced by TTP was identical with that produced by GTP or ITP (Fig. 7), i.e., the effect shows little nucleotide specificity. This nonselective effect of nucleoside triphosphates on the binding of labeled TSH is in contrast to the direct correlation observed at lower concentrations between the ability of the nucleotides to displace PGE₁ (50% displacement at 2 × 10⁻⁹ M nucleotide) and their ability to enhance PGE₁ activation of adenylyl cyclase in these membranes (18).

TSH Binding under Conditions of Adenylyl Cyclase Assay—In order to compare binding of TSH to its effects on adenylyl cyclase it was important to investigate TSH binding under the temperature and medium conditions used for cyclase measurements. Membrane protein concentrations are approximately one-third those used under “binding” conditions. Each of the components of the cyclase assay medium was evaluated for its effect on TSH binding. Mg²⁺, creatine phosphate, and ATP each decreased binding by about one-half (Table I); however, the inhibitions were not additive and TSH binding was reduced to about one-third of control binding in the complete cyclase medium. However, TSH binding to the membranes in this medium for 10 min at 37°C was sufficient to permit accurate...
The binding reaction was initiated by adding 30 μg of membrane protein to the incubation media at 37° containing 750 picomoles per ml of TSH, 25 mM Tris-HCl, 0.3 mM ITP plus the component listed in the table, final volume 0.120 ml. The mixture was incubated for 10 min at 37° and then layered on the discontinuous gradient at 37° and centrifuged at 40,000 x g for 10 min at 2.5°. The complete mixture contained all of the components at the listed concentrations.

| Additions            | Concentration | TSH bound |<|p|moles/mg protein ± S.D.|
|---------------------|---------------|-----------|
| Tris-HCl, pH 7.6    | 25            | 145 ± 8   |
| +cAMP               | 0.3           | 147 ± 10  |
| +Mg²⁺              | 2.0           | 64 ± 11   |
| +Creatine phosphate | 14            | 76 ± 9    |
|                     | 7             | 89 ± 3    |
|                     | 3.5           | 136 ± 10  |
|                     | 1.0           | 69 ± 7    |
| Complete mixture    |               | 55 ± 4    |

The binding reaction was initiated by adding the membrane protein to the appropriate assay medium containing the activator or inhibitor and 0.7, 900, or 750 nM TSH. The 0.7 and 900 nM TSH concentrations were used to determine binding under “binding” conditions and 750 nM TSH was used under cyclase conditions. The incubation time was 15 and 10 min for binding and cyclase conditions, respectively, and incubation temperature was 0-2, 22-24, and 37° for the 0.7, 900, and 750 nM TSH concentration assay, respectively. Several membrane preparations were used for the “binding” condition assay giving final membrane protein concentrations of 1.09 to 1.89 mg per ml. The membrane protein concentration was 0.3 mg per ml in the cyclase condition assay. Binding was terminated by layering the incubation solution on a microfuge gradient at the respective temperature and centrifuging at 40,000 x g for 10 min at 4-5°. Results are expressed as per cent of control binding. The TSH bound for the 0.7, 900, and 750 nM TSH concentration was 0.71 to 0.38, 93 to 38, and 62 to 45 pmoles per mg of membrane protein, respectively. The effect on TSH binding at an agent concentration which produced 50% inhibition of TSH-stimulated cyclase is identical even though the response at a higher concentration is listed.

<table>
<thead>
<tr>
<th>Agent and concentration</th>
<th>Binding condition 0.7 nM TSH specific binding</th>
<th>Cyclic condition 900 nM TSH specific binding</th>
<th>TSH-stimulated adenylate cyclase (750 nM TSH specific binding)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Concentration for 50% inhibition</td>
<td>Concentration</td>
<td>&lt;</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Promacine (0.1 mg/ml)</td>
<td>7.6</td>
<td>5.3</td>
<td>35.4</td>
</tr>
<tr>
<td>Cembranone (1.6 x 10⁻⁴ m)</td>
<td>65.2</td>
<td>73.6</td>
<td>66.6</td>
</tr>
<tr>
<td>Phospholipase A (7.0 µg/ml)</td>
<td>153.5</td>
<td>162</td>
<td>0.16 µg/ml</td>
</tr>
<tr>
<td>Filipin (5 x 10⁻⁴ m)</td>
<td>191</td>
<td>193</td>
<td>3 x 10⁻⁴</td>
</tr>
<tr>
<td>Chlorpromazine (0.3 mm)</td>
<td>105</td>
<td>103</td>
<td>2 x 10⁻⁴</td>
</tr>
<tr>
<td>Valinomycin (1 x 10⁻⁸ m)</td>
<td>105.2</td>
<td>103</td>
<td>4 x 10⁻⁴</td>
</tr>
<tr>
<td>Gramicidin S (1 x 10⁻⁸ m)</td>
<td>96.8</td>
<td>94.0</td>
<td>96.6</td>
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<tr>
<td>Cetyl pyridinium chloride (5 x 10⁻⁴ m)</td>
<td>103.1</td>
<td>103</td>
<td>1 x 10⁻⁵</td>
</tr>
<tr>
<td>PGF₂α (2 x 10⁻⁴ m)</td>
<td>109</td>
<td>116</td>
<td>100</td>
</tr>
<tr>
<td>Prostaglandin A (2.8 x 10⁻⁸ m)</td>
<td>90.8</td>
<td>99.2</td>
<td>90.4</td>
</tr>
<tr>
<td>Indomethacin (0.6 mm)</td>
<td>101</td>
<td>109</td>
<td>89.4</td>
</tr>
</tbody>
</table>

Since PGF₂α is a fatty acid that is known to bind to thyroid membranes with high affinity (18), it has been claimed to interfere with the full expression of TSH stimulation of adenylate cyclase (31), its effect on TSH binding in the various media was observed under cyclase conditions (Fig. 3) with apparent affinities of 1.6 x 10⁻⁶ and 0.83 x 10⁻⁶ M at 37° and respective capacities of 12.7 and 175 pmoles per mg of membrane protein. These apparent affinities correspond to the affinities of the two binding sites measured under “binding” conditions; however, the higher affinity constant was reduced by an order of magnitude due to the cyclase medium, temperature, etc., whereas the second site was not substantially affected. Reference to Fig. 3 reveals that these two apparent binding constants correspond roughly to the two zones of activation of adenylate cyclase that exhibit midpoints at 1 x 10⁻⁷ and 1 x 10⁻⁸ M, respectively.

The effect of ITP and GTP on TSH binding was identical with that observed under “binding” conditions (Fig. 7C). Apparently, the presence of a regenerating system is not important for nucleotide effects on TSH binding to these membranes, in contrast to other systems (23, 24).

**Effect of Inhibitors of Adenylate Cyclase**—We have shown previously (6, 7, 13) that thyroid membrane adenylate cyclase is readily inhibited by a variety of agents believed to react with the membrane. Chlorpromazine, gramicidin S, and cobramine B inhibit TSH-activated adenylate cyclase at concentrations of 10⁻⁹ to 10⁻⁶ M, whereas 10-fold higher concentrations stimulate F⁻-activated cyclase. At still higher concentration, F⁻-stimulated activity is inhibited as well. Filipin, cetylpyridinium chloride, and valinomycin inhibit TSH-stimulated and F⁻-stimulated adenylate cyclase but do not cause enhancement of F⁻-stimulated activity (6, 13) at intermediate concentrations.

The ability of beef thyroid membranes to bind TSH was not altered by chlorpromazine, gramicidin S, valinomycin or cetylpyridinium chloride at concentrations that caused 50% inhibition of TSH-activated adenylate cyclase activity (Table II). For this reason these inhibitors were tested at 10-fold or greater concentrations, but still no effect was observed on TSH binding, irrespective of the TSH concentration or the binding conditions listed (Table II). In contrast, filipin, which interacts with membrane sterols and possibly phospholipids (25), not only failed to block TSH binding, but increased binding at concentrations of 50 and 500 µM but not at 5 µM.

Because the filipin results suggested a possible role for lipids in TSH binding, we investigated the effect of phospholipase A from cobra venom. It has been shown previously that pure phospholipase C (26) and phospholipase A (27, 28) profoundly depress the stimulation of adenylate cyclase by TSH. Treatment of beef thyroid membranes with pure phospholipase A resulted in marked inhibition of the TSH response of the cyclase (Fig. 8). As little as 30 ng per ml of phospholipase A caused significant inhibition of cyclase activity. When thyroid membranes were preincubated with 7 µg per ml of phospholipase A, no inhibition of TSH binding could be observed, and, in fact, there was a slight but consistent increase in binding. Increased hormone binding has also been obtained with phospholipase A in liver membranes binding glucagon (29) and fat cell membranes binding insulin (30). Whether or not this increase implies that “buried” receptors become exposed, or whether the nature of the binding site is altered, remains to be determined.
The membranes were incubated in the presence of 1.0% bovine serum albumin, 3.5 mM CaCl₂, 5 mM Tris-HCl buffer (pH 7.7), and the reaction was started by addition of pure phospholipase A. Reactions were run at 23°C for 10 min and were stopped by addition of 0.1 mM EGTA to a final concentration of 4.3 mM. The solution was cooled to 0°C and aliquots were immediately used for the assay of adenylate cyclase. Controls were run without enzyme or by addition of EGTA at the start. All points are the means of triplicates.

In contrast to agents that affect membrane lipids, those that alter membrane proteins interfere with TSH binding. Under all binding conditions preincubation of membrane with 100 μg per ml of pronase severely inhibited the binding of TSH. This was not compensated for by a corresponding increase in the nonspecific TSH bound. Adenylate cyclase activity was completely destroyed by similar treatment. We believe that this inhibition is not due to hydrolysis of TSH by pronase, since (a) >90% of the enzyme was removed by centrifugation of the membranes before assay, (b) binding assays were carried out in serum albumin, and (c) binding at 0°C was inhibited to the same extent as binding at 22°C (Table II).

Cobramine B, a small basic protein obtained from cobra venom, has powerful effects on the thyroid membrane including the induction of leakiness for K⁺ and I⁻ (32), and inhibition of adenylate cyclase (6). It caused significant inhibition of TSH binding under all conditions but this effect was less sensitive than the inhibition of TSH-stimulated adenylate cyclase (Table II). The importance of using purified phospholipase A is clearly indicated by our results with cobramine B. Since this protein of cobra venom survives the heating step usually used for the "purification" of phospholipase A, an effect attributed to phospholipid hydrolysis by such a preparation might, in fact, be due to cobramine B.

**DISCUSSION**

The adenylate cyclase system has generally been divided into three domains: that of the receptor for hormones, that of the catalytic activity, and the connection between these, referred to as the transducer or coupling process (33). Whether or not these domains are regulatory components of a complex allosteric system remains to be established. Nevertheless, it is possible to identify the domain where a number of modulators or regulators may act by comparing the effect of such agents on the binding of hormone with their effects on cAMP production.

It was first necessary to characterize TSH binding to the thyroid membranes used for cyclase studies. Both H-labeled and I²-Th-TSH bound reversibly and rapidly to these membranes, and under identical conditions, binding was independent of the nature of the label used. We were able to identify at least two separate binding sites (under "binding" conditions) with apparent affinity constants of 2.2 to 3.1 × 10⁶ M⁻¹ at 2°C and 0.08 to 1.1 × 10⁶ M⁻¹ at 23°C. A number of the properties of these sites are similar to those reported by Amir et al. (5). Similar binding properties are also seen in Harderian gland membranes (34) and human peripheral lymphocytes. When binding of TSH was measured under the medium and temperature conditions used in the adenylate cyclase assay, there was a considerable decrease in specific binding. The apparent high affinity constant was reduced to 1.6 × 10⁶ M⁻¹ whereas the second constant showed little alteration. The number of sites with the high affinity constant was increased approximately 7-fold.

As pointed out above, the affinity constants are rather low when compared to effective TSH concentrations in vitro. Whether or not their numerical value is reduced as the result of the preparative procedure or storage, as occurs with the angiotensin II receptor of adrenal particles (34), remains to be determined. It is also important to point out that such apparent constants must be interpreted with caution since it has not been definitely established that first order analysis is appropriate to the binding system. Moreover, binding is extremely sensitive to the components of the medium. Thus the above constants may be applicable when comparing binding to adenylate cyclase activation in isolated membranes but may well have different values when such comparisons are made in tissue slices, in isolated cells or in vivo.

Despite the differences in the apparent affinity constants of the thyroid membranes for TSH, the activators and inhibitors of adenylate cyclase affected binding identically at both binding sites and independently of the TSH concentration and media. The modulators can be classified into four groups according to their effects on TSH binding (Table III): (a) agents which affect TSH-activated adenylate cyclase and TSH binding in the same direction; (b) agents which enhance TSH activation of adenylate cyclase and inhibit binding; (c) agents which inhibit TSH activation of adenylate cyclase and stimulate TSH binding; and (d) agents which affect TSH-activated adenylate cyclase but do not affect binding. The number of agents that exhibit a direct correlation between adenylate cyclase activity and TSH binding is small. The cations Ca²⁺, Na⁺, Li⁺, and cobramine B inhibited both cyclase activity and TSH binding under all medium conditions. In the case of Ca²⁺, Na⁺, and cobramine B, the inhibition concentrations were of the same order, although cyclase tended to be more sensitive. On the other hand, concentrations of Li⁺ that yielded 50% inhibition of adenylate cyclase (11) did not affect binding of TSH. At much larger concentrations, Li⁺ did interfere with TSH binding (Fig. 4) but, in contrast to the effect on cyclase, its potency was smaller than that of Na⁺. It seems probable, therefore, that the Li⁺ effect on adenylate cyclase does not result from inhibition of TSH binding.

The locus of interaction of the next two classes of compounds with the cyclase system is more difficult to specify. Whether

\[ \text{FIG. 8. Effect of phospholipase A on adenylate cyclase activity.}\]

The membranes were incubated in the presence of 1.0% bovine serum albumin, 3.5 mM CaCl₂, 5 mM Tris-HCl buffer (pH 7.7), and the reaction was started by addition of pure phospholipase A. Reactions were run at 23°C for 10 min and were stopped by addition of 0.1 mM EGTA to a final concentration of 4.3 mM. The solution was cooled to 0°C and aliquots were immediately used for the assay of adenylate cyclase. Controls were run without enzyme or by addition of EGTA at the start. All points are the means of triplicates.
the enhancement of cyclic AMP production; even 1000-fold greater nucleotide concentrations did not alter TSH binding at concentrations that compared at equivalent agent concentration.

Despite their potent effects on adenylate cyclase, nucleoside diphosphates did not alter TSH binding at concentrations that increased hormone binding cannot be stated from the present data. In contrast, agonists that promote TSH binding without concomitant stimulation of adenylate cyclase may act on receptors that are not coupled efficiently to the remainder of the system. Alternatively, certain alterations in the transducer may cause increased TSH binding without concomitant activation of cyclase. The fact that phospholipase A, for example, inhibits F- and PGE2-activated cyclase suggests that binding changes may be secondary to changes in the domain of the coupling reaction and not primarily on the TSH receptor domain (23). Members of the last category inhibit TSH-stimulated adenylate cyclase at a stage beyond TSH binding. The fact that inhibitors like chlorpromazine, gramicidin S, valinomycin, and cetyl pyridinium chloride may stimulate F-activated cyclase activity is associated with dissociation of hormone from its receptor (22), in which a regenerating system is required (23), and in which GTP is more potent than TTP (23, 24); and (b) that represented by thyroid, and possibly turkey erythrocyte membranes (35), where no dissociation is induced by nucleotides, where a regenerating system appears to have little effect, and where TTP is more potent than GTP (7, 35). Since these nucleotides do displace PGE2 from thyroid membranes (18), the effect may be more closely associated with a particular receptor pathway than with the membrane as a whole.

In conclusion it appears that the two TSH binding sites of beef thyroid membranes have many properties that are similar despite large differences in TSH concentrations that have to be used to elicit measurable responses at these sites; thus studies over a considerable range of TSH concentrations are likely to be physiologically meaningful. On the other hand, TSH binding appears to be relatively insensitive to many of the activators and inhibitors of adenylate cyclase, and their intervention in this system occurs after the binding stage.

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