Inhibition of Thyroid-Stimulating Hormone Stimulation of Protein Kinase, Glucose Oxidation, and Phospholipid Synthesis in Thyroid Slices Previously Exposed to the Hormone

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ABSTRACT Prior exposure of thyroid slices to thyrotropin (TSH) induced refractoriness to subsequent stimulation of the cyclic AMP system by the hormone. Although the inhibition is incomplete, we examined whether the reduction in cyclic AMP was sufficient to alter other metabolic effects of TSH. Bovine or dog thyroid slices were incubated with or without 5-100 mU/ml TSH for 1-2 h, washed, and then incubated without hormone for 1-2 h. Half of the slices not exposed to TSH initially were then incubated with buffer and half were exposed to 5-100 mU/ml TSH. Slices initially incubated with TSH were also incubated with or without TSH in the third incubation. During the refractory period, TSH activation of protein kinase was inhibited even though the hormone still caused some increase in cyclic AMP concentrations. However, protein kinase activity was fully responsive to dibutyl cyclic AMP when slices were incubated with it during the third incubation. Stimulation of glucose oxidation by TSH was significantly decreased in thyroid slices previously incubated with the hormone. During refractoriness, stimulation of glucose oxidation caused by prostaglandin E₁ and dibutyl cyclic AMP was also significantly diminished but that due to acetylcholine was not. Thus even though dibutyl cyclic AMP could fully activate protein kinase activity during refractoriness, its effect on glucose oxidation was still inhibited, suggesting that the metabolic block responsible for this refractoriness was distal to activation of protein kinase. Stimulation of 32P₁ incorporation into phospholipid by TSH and acetylcholine was also inhibited during refractoriness. Despite reduction of the stimulatory effect of TSH, binding of 125I-TSH was not modified by prior incubation of thyroid slices with TSH. These results indicate that changes in the TSH receptor are not responsible for the development of refractoriness and other metabolic sites besides activation of adenylate cyclase appear to be involved.

INTRODUCTION

We previously reported that the cyclic 3', 5'-adenosine monophosphate (cyclic AMP) response to thyrotropin (TSH)¹ was significantly reduced in thyroid slices previously exposed to the hormone (1). Refractoriness required at least a 30-min initial exposure to TSH and was not reversed by a subsequent 5-h incubation of the slices in the absence of hormone. Refractoriness was not dependent on new protein synthesis nor on release of either iodide or thyroid hormone. During the refractory period, TSH did not stimulate adenylate cyclase activity. The observation that NaF activation of adenylate cyclase was unimpaired suggested that the refractoriness reflected an alteration in either the binding or the coupling step. Diminished responsivity of adenylate cyclase to stimulation by the appropriate hormone has also been reported in other tissues during the refractory period (2–6).

During the refractory period in the thyroid, the augmentation of cyclic AMP by TSH is not totally inhibited (1). Whereas maximum biological effects of

¹Abbreviations used in this paper: ACH, acetylcholine; DBC, dibutyl cyclic AMP; PGE₁, prostaglandin E₁, TSH, thyrotropin.


TSH on a variety of metabolic parameters can be observed at concentrations of TSH which do not stimulate cyclic AMP maximally (7), the present studies investigated whether the reduction in cyclic AMP was sufficient to alter some of these other effects of TSH. In addition, binding of $^{125}$I-TSH to homogenates of thyroid slices was measured to ascertain if the failure of TSH to activate adenylate cyclase during the refractory period correlated with changes in binding.

**METHODS**

Experiments were done using both beef and dog thyroid tissue obtained, sliced, and incubated as previously reported (1). Beef thyroid tissue was used for studies of activation of protein kinase and binding of $^{125}$I-TSH because such studies required more tissue than could be obtained from a dog thyroid. The latter tissue was more sensitive to stimulation of glucose oxidation and phospholipid synthesis by TSH and the other agents tested. Unless otherwise stated, three sequential incubations were done. Slices were first incubated (1–2 h) in a Dubnoff metabolic shaker at 37°C in 2 ml Krebs-Ringer bicarbonate buffer containing 1 mg/ml glucose and 1 mg/ml bovine serum albumin with or without TSH. Concentrations of TSH varied because the sensitivity of the various metabolic parameters to TSH is different (7). After the initial incubation, slices were rinsed in excess 0.85% saline and then incubated for 1–2 h in 2 ml Krebs-Ringer bicarbonate buffer containing 1 mg/ml glucose. Slices were then rinsed in 0.85% saline, blotted on filter paper, and weighed. After this, some of the slices, both from the control and TSH group, were homogenized in 0.001 M Na bicarbonate buffer for determination of binding of $^{125}$I-TSH as described subsequently. The remainder of the slices were transferred to fresh buffer containing 1 mg/ml glucose and 1 mg/ml albumin and incubated for a third incubation of 20 min when cyclic AMP was measured. Of the control slices incubated in buffer without TSH in the first incubation, half were again incubated in buffer without TSH, and the other half were exposed to TSH for the first time. Similarly, of the slices incubated with TSH during the initial incubation, half were now incubated without TSH and the other half were re-exposed to the hormone. When glucose oxidation was measured, [1-$^{14}$C]glucose was added to the buffer during the third incubation, and the TSH concentration was 5 mU/ml. Incubation was for 45 min and $^{14}$CO$_2$ production was determined as previously described (8). The effect of dibutyryl cyclic AMP (DBC), prostaglandin E$_1$ (PGE$_1$) and acetylcholine (ACH) on glucose oxidation in thyroid slices refractory to TSH was assessed by adding these substances during the third incubation to slices which had been incubated with and without TSH during the first incubation. When $^{32}$P$_i$ incorporation into phospholipid was measured 10 μCi/ml Na $^{32}$PO$_4$ was added during the third incubation of 2 h. Incorporation of $^{32}$P$_i$ into phospholipids was determined as previously described (9). Acetylcholine was added during the third incubation to some slices which had been incubated with and without TSH during the initial incubation. When acetylcholine was used, 0.4 mM eserine was also present in the buffer.

Effects of TSH and DBC on activation of protein kinase were assessed during the third incubation (20 min) by adding the substance to appropriate thyroid slices which had been incubated with and without TSH during the first incubation. After the third incubation, slices were homogenized and assayed for protein kinase activity in the presence and absence of 1 μM cyclic AMP (10) and for cyclic AMP (11). Theophylline was not present during the third incubation.

Binding of $^{125}$I-TSH to homogenates obtained from thyroid slices after the second incubation was measured by the method of Kotani et al. (12) using microcentrifugation to separate the bound and free hormone. The homogenate was prepared by the method of Manley et al. (13). The results are corrected for nonspecific binding which was measured in the presence of 100 mU/ml TSH and was less than 20% of the total binding. Under the conditions

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**TABLE I**

**Effects of TSH and DBC on Protein Kinase Activity of Beef Thyroid Slices Previously Exposed to TSH**

<table>
<thead>
<tr>
<th>Protein kinase activity</th>
<th>Addition to third incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>5 mU/ml TSH</td>
</tr>
<tr>
<td></td>
<td>12.5 μg/ml DBC</td>
</tr>
<tr>
<td></td>
<td>125 μg/ml DBC</td>
</tr>
<tr>
<td><strong>Addition to first incubation</strong></td>
<td>-CAMP/</td>
</tr>
<tr>
<td>None</td>
<td>2.4±0.3</td>
</tr>
<tr>
<td>TSH, 5 mU/ml</td>
<td>1.6±0.1</td>
</tr>
</tbody>
</table>

* P < 0.01 compared to 0.42±0.01.
† P < 0.02 compared to 0.56±0.01.

Beef thyroid slices weighing between 60 and 90 mg were incubated with and without 5 mU/ml TSH during the first incubation for 2 h. The second incubation in buffer alone was also for 2 h. The third incubation was for 20 min in the presence of buffer, 5 mU/ml TSH or 12.5 or 125 μg/ml DBC as indicated. Theophylline was not present during the third incubation. Slices were homogenized at the end of the third incubation in 0.05 M sodium glycerophosphate buffer (pH, 6.5) containing 10 mM MgCl$_2$, 10 mM NaF, 2 mM theophylline and 0.3 mM EGTA. The homogenate was centrifuged at 12,000 g for 15 min and the supernate was used for protein kinase assay. The protein kinase activity was measured with and without 1 μM cyclic AMP for 5 min with 100 μg histone as substrate. Other slices were extracted in hot 50 mM sodium acetate buffer (pH, 4.0) for the end of the third incubation for measurement of cyclic AMP. The cyclic AMP concentration in slices never exposed to TSH was 166±24 pmol/g compared to 980±96 pmol/g in slices exposed to 5 mU/ml TSH only during the third incubation. The cyclic AMP concentration in slices exposed to 5 mU/ml TSH in only the first incubation was 32.32 pmol/g and was significantly (P < 0.01) increased by 65±17 in slices exposed to TSH during both the first and third incubations. The last value is significantly less (P < 0.05) than the value of 980±96 indicating refractoriness to TSH. The cyclic AMP and protein kinase results are the mean±SEM of triplicate determinations.
of the experiment, no loss of TSH or its receptors was apparent (12).

TSH (NIH-B6, 2.5 U/mg) was kindly provided by the National Institute of Arthritis, Metabolism and Digestive Diseases. PGE, was a gift from Dr. John Pike, The Upjohn Co., Kalamazoo, Mich. DBC was purchased from Sigma Chemical Co., St. Louis, Mo. and ACH from Merck Chemical Div., Merck & Co., Inc., Rahway, N. J. 24.1 Ci/mmol cyclic [H]AMP and 50–1,000 mCi/mmol Na₂³¹PO₄ were obtained from New England Nuclear, Boston, Mass. 1.14 Ci/mmol ³²P⁺[³²P]ATP and 2.9 mCi/mmol [¹³C]glucose were purchased from Amersham/Searle Corp., Arlington Heights, Ill. Dr. John Pierce, University of California, Los Angeles, generously provided 30 U/ml purified bovine TSH which was iodinated with ¹²⁵I by the lactoperoxidase method (12).

RESULTS
The data in Table I demonstrate that prior incubation of thyroid slices with TSH inhibits the subsequent ability of the hormone to activate protein kinase activity. The enzyme activity assayed without cyclic AMP was 2.4±0.3 pmol ³²P incorporated/mg of tissue per 5 min in slices never exposed to TSH and 3.2±0.2 in slices incubated with TSH only during the third incubation. The total enzyme activity (assayed with 1 μM cyclic AMP) was 5.7 pmol ³²P incorporated/mg of tissue per 5 min whether or not TSH was present during the third incubation. In slices not exposed to TSH in the first incubation, the protein kinase activity ratio (—cyclic AMP/+cyclic AMP) was significantly increased (P < 0.01) from 0.42±0.01 to 0.56±0.01 by the addition of TSH during the third incubation. The lack of a greater increase in the activity ratio can be attributed to the relatively modest increase in the cyclic AMP concentration (basal, 186±24 pmol/g; TSH, 980±96 pmol/g) induced by TSH in the absence of theophylline (10). In contrast, 125 μg/ml DBC almost completely activated protein kinase (activity ratio 0.85±0.05). The smaller amount of DBC (12.5 μg/ml) induced less activation of protein kinase. In more extensive experiments (data not shown) this smaller amount of DBC was demonstrated to be less than maximal. The protein kinase activity of slices incubated with TSH during the first incubation only was decreased whether the assay was done with or without 1 μM cyclic AMP (1.6±0.1 and 3.8±0.2, respectively). Despite such reduction, the protein kinase activity ratio (0.43±0.04) was the same as in slices which had never been incubated with TSH. The addition of TSH to slices which had been incubated with the hormone in the first incubation did not augment protein kinase activity and the activity ratio was 0.45±0.02, significantly less (P < 0.02) than the value of 0.56±0.01 obtained in slices exposed to TSH only in the third incubation. Although the protein kinase was unresponsive to readdition of TSH during the refractory period, the stimulation by both a submaximal and maximal amount of DBC was unimpaired. The cyclic AMP concentration at the end of the third incubation in slices exposed to TSH only during the first incubation was 252±32 pmol/g of tissue and significantly increased (P < 0.01) to 658±17 when TSH was again added during the third incubation. This latter value was significantly less (P < 0.05) than the value of 980±96 found in slices incubated with TSH only during the third incubation.

During the refractory period, the effect of TSH on glucose oxidation was also significantly decreased (Fig. 1). In this experiment, basal glucose oxidation was reduced during the third incubation in slices exposed to 25 mU/ml of TSH during the first incubation.
inhibited when it was added to thyroid slices which had been incubated with TSH during the first incubation. Stimulation of glucose oxidation in dog thyroid slices by 1μM acetylcholine (14) was not altered by incubating slices with TSH during the first incubation.

The results in Fig. 2 indicate that stimulation by TSH of 32P₁ incorporation into phospholipids is also significantly (P < 0.01) diminished during the refractory period induced by prior exposure of tissue to the hormone. 100 μU/ml TSH added during the third incubation doubled 32P₁ incorporation into phospholipid in dog thyroid slices not previously exposed to the hormone. However, the effect of TSH was significantly diminished when it was added to slices exposed to the hormone during the first incubation. In contrast to the results obtained when glucose oxidation was measured, the stimulatory effect of acetylcholine on 32P₁ incorporation into phospholipids (9) was decreased in thyroid slices exposed to TSH during the first incubation.

Our previous finding that TSH, but not NaF, stimulation of adenylate cyclase was decreased in tissue previously incubated with the hormone (1), suggested that refractoriness might reflect an alteration in either TSH binding to its receptors or in the transmission of the signal to the catalytic unit of adenylate cyclase. The data in Fig. 3 demonstrate that prior incubation of thyroid slices with TSH did not diminish TSH binding by homogenates prepared at the end of the second incubation. Furthermore, displacement of 125I-TSH from its binding sites in both homogenates was similar when 2–25 μU/ml of unlabeled TSH was added.

**DISCUSSION**

The present studies extend the previous observation that initial exposure of thyroid tissue to TSH induces diminished responsiveness upon subsequent addition of the hormone (1). During the refractory period, activation of protein kinase by TSH was inhibited despite a significant, but blunted, increase in cyclic AMP concentration. According to our previous results (reference 10, Table V), even this decreased amount of cyclic AMP should have been sufficient to cause some activation of protein kinase. This might suggest that the enzyme was refractory, but this is not supported by the undiminished activation of it induced by addition of DBC to slices during the third incubation. A submaximal amount of DBC was deliberately used in case there was an alteration in sensitivity of protein kinase rather than total unresponsiveness. The results suggest that the increased cyclic AMP generated by TSH in the slices previously exposed to the hormone was not

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**FIGURE 2** Effect of TSH and acetylcholine (ACH) on 32P₁ incorporation into phospholipids in dog thyroid slices previously incubated with and without 100 μU/ml TSH. Dog thyroid slices (20–40 mg) were incubated with and without TSH for 2 h and then incubated without hormone for 2 h. During the third incubation of 2 h, 10 μCi, 7 × 10⁶ cpm Na₃₂PO₄ was added and the P₁ concentration was 0.0012 M. The total incubation volume was 2 ml. Test substances were present as indicated. The results are the mean±SEM of triplicate slices. (*)P < 0.01 when compared to slices which had never been exposed to TSH. (**)P < 0.01 when compared to slices which had been initially incubated with TSH. (+)P < 0.01 when compared to its appropriate control which had never been exposed to TSH.
available for activation of protein kinase. It is also possible that TSH during the first incubation induced an inhibitor of protein kinase which prevented activation of the enzyme by the increased cyclic AMP due to TSH added in the third incubation. Such an inhibitor could have been overcome by the larger amounts of DBC which were present in the third incubation. If such an inhibitor were produced, it obviously did not interfere with the stimulation of protein kinase by 1 μM cyclic AMP added to the homogenate during the assay of the enzyme. Dilution of the homogenate for the assay could explain this. Thus the refractoriness of protein kinase could reflect the previously demonstrated diminished responsiveness of adenylate cyclase (10) in that insufficient cyclic AMP was available to facilitate dissociation of the enzyme into its regulatory and catalytic subunits. The lower levels of cyclic AMP in the present experiments reflect the absence of theophylline from the third incubation. This was done to make the incubation conditions more comparable to those for glucose oxidation and phospholipid synthesis since theophylline was not included in those incubations. We previously reported that incubation of thyroid slices with TSH sometimes decreased the total amount of protein kinase activity (10). Although this was not observed in the experiment in Table I in the slices incubated without TSH in the first incubation, it probably accounts for the reduced amounts of enzyme in the slices which had been incubated with TSH during the initial incubation. It is not apparent why addition of TSH or DBC to these latter slices during the third incubation did not cause further reduction in total protein kinase activity.

The results measuring glucose oxidation indicate

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that the basis for refractoriness is more complex than unresponsiveness of adenylate cyclase. In dog thyroid slices, stimulation of glucose oxidation by TSH (7) and PGE, (15) probably involves the adenylate cyclase-cyclic AMP system. During the refractory period induced by TSH, the effect of PGE, on cyclic AMP was not diminished (1), but its action on glucose oxidation was significantly reduced. The results with DBC provide even more convincing data that a more distal site than activation of protein kinase is involved in refractoriness. Thus DBC was capable of fully activating protein kinase during the refractory period but it did not augment glucose oxidation. These observations also exclude binding of TSH as the only locus for induction of refractoriness. PGE, has specific receptors on the thyroid (16) which are independent of TSH receptors (12, 16). The failure to detect any change in binding of 125I-TSH to partially purified plasma membranes prepared from slices refractory to TSH provides more direct evidence that alteration of the TSH receptor site is not involved. However, it is possible that such changes in the TSH receptors in the slice were lost during the preparation of the partially purified membranes. Mills and McPherson (17) also reported that binding of the luteinizing hormone to ovarian follicles was unchanged when that tissue was refractory to the hormone. Furthermore, numerous examples have been reported where there has been dissociation of the binding of TSH to its receptors and effects on activation of adenylate cyclase activity (12, 18).

The stimulation of glucose oxidation by acetylcholine (14) was not inhibited in slices which were refractory to TSH. This observation is consistent with the involvement of the adenylate cyclase-cyclic AMP system since acetylcholine does not exert its effects via this mechanism (19). Although the mechanism by which acetylcholine stimulates glucose oxidation is not known, the present results would indicate that it probably does not share much of a common pathway with the effect of TSH, PGE,, or DBC.

Inhibition of the TSH stimulation of 32P incorporation into phospholipids provides further support for involvement of processes other than activation of adenylate cyclase as the cause of refractoriness. Much evidence has been provided indicating that this effect of TSH is not mediated by the adenylate cyclase-cyclic AMP system (7, 20, 21). Therefore, a single defect in activation of adenylate cyclase would not explain development of refractoriness to TSH when 32P incorporation into phospholipids is measured. Furthermore, stimulation of 32P incorporation into phospholipids by acetylcholine was also decreased in thyroid slices previously incubated with TSH. This is in contrast to the results obtained when glucose oxidation was measured since TSH did not induce refractoriness to acetylcholine in this parameter. This provides further support for the dissociation of effects of TSH and acetylcholine. The results would be compatible with acetylcholine and TSH stimulation of 32P incorporation of phospholipids having some common intermediate which would then be a site of the refractoriness.

Although these studies clearly demonstrate that unresponsiveness to TSH extends to other metabolic effects of the hormone in addition to generation of cyclic AMP, they do not provide unequivocal evidence that such refractoriness is of physiological significance or involved in the hormonal regulation of thyroid gland function.

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REFERENCES


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