Activation of Adenyl Cyclase in Thyroid Homogenates by Thyroid-Stimulating Hormone

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It has been proposed that cyclic 3',5'-AMP mediates the effect of thyroid-stimulating hormone (TSH) on the thyroid. The evidence in support of this hypothesis is as follows: a. TSH increases the levels of cyclic 3',5'-AMP in thyroid slices (Gilman and Rall, 1966); and b. dibutyryl cyclic 3',5'-AMP, an analogue of cyclic 3',5'-AMP, reproduces in thyroid slices the effects of TSH on glucose oxidation, phospholipid synthesis and the induction of pseudopods and intracellular colloid droplets (Pastan, 1966; Pastan and Wollman, 1967). The present paper shows that TSH added to thyroid homogenates increases the rate of synthesis of cyclic 3',5'-AMP.

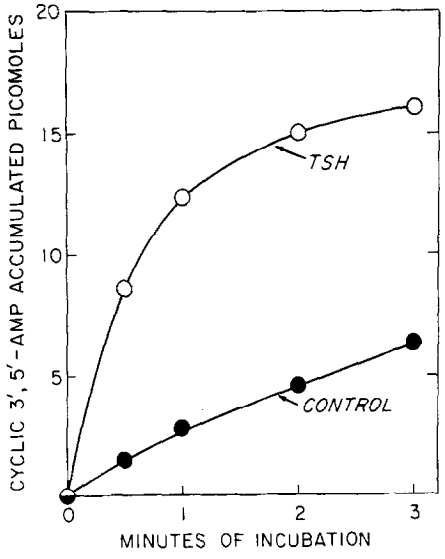
Materials and Methods. Bovine TSH (4 I.U./mg and 25 I.U./mg) was a gift of Dr. Peter Condliffe, National Institutes of Health, crystalline porcine insulin (25 U/mg) a gift of Eli Lilly, bovine growth hormone (4 U/mg, F-II-286) a gift of Dr. Martin Sonnenberg, New York, and human chorionic gonadotrophin (2,671 U/mg) a gift of Dr. Aleck Borman, Squibb Institute. Adenosine 5'-triphosphate disodium salt (ATP) was purchased from P-L Biochemicals, AT^{32}P (α-labelled at 550mC/mMole) from International Chemical and Nuclear Corporation, ^{3}H-cyclic 3',5'-AMP (1C/mMole) from Schwarz Bio-

research cyclic 3',5'-AMP and Dowex 50W-X8, 100-200 mesh from Calbiochem, epinephrine-HCl from Parke-Davis, crystalline bovine serum albumin from Armour, porcine adrenocorticotrophic hormone (ACTH) at 140U/mg from Sigma and theophylline and acetylcholine from Mann.

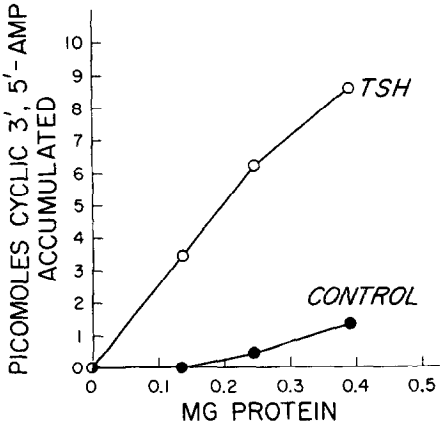
Bovine thyroids were obtained from an abattoir and dog thyroids from exsanguinated animals. The tissues were sliced and about 300 mg wet weight of slices were homogenized in 1.0 ml of cold 0.25M sucrose in a Dounce homogenizer (Kontes). Adenyl cyclase activity was measured by a recently described method (Krishna, Weiss, and Brodie, 1967; Weiss and Costa, 1967; Rodbell, 1967; Taunton, Roth and Pastan, 1967). Each 0.06 ml reaction mixture contained ATP, 1.3 mM; 2-3x10^6 cpm of α-labelled $AT^{32}P$, theophylline, 8 mM; $MgCl_2$, 2 mM; tris-Cl, 21 mM; 0.08% albumin and variable amounts of thyroid homogenate. The pH was 7.5 and the temperature was 37°C. Reactions were initiated by adding homogenate which was kept at 1°C to the other components which were kept at 23°C to dissolve the theophylline. Hormones were added to the homogenate just before reactions were initiated. Reactions were stopped by placing the tubes in boiling water for three minutes. Just prior to heating 0.1 ml of a solution containing 4 μmoles of ATP, 1.25 μmoles of cyclic 3',5'-AMP and 0.15 μcuries of ^{3}H-cyclic 3',5'-AMP was added. The ^{3}H-cyclic 3',5'-AMP served to determine the recovery of the cyclic 3',5'-AMP during the subsequent steps; recoveries were 30-40%. After heating, water (0.4 ml) was added, the precipitate was removed by centrifugation and the supernate was passed over a 0.5 x 2.0 cm Dowex-50 column in the H^+ form. The column was washed with water and the cyclic 3',5'-AMP emerged between 3.0-6.0 ml. Trace quantities of other nucleotides and phosphate were precipitated by the addition of 0.2 ml of 0.17M $ZnSO_4$ followed by 0.2 ml of 0.15M $Ba(OH)_2$. The $BaSO_4$ was removed by centrifugation and the precipitation step repeated. An aliquot of the supernate was added to 17 ml of Bray's solution and the radioactivity measured in a liquid-scintillation

spectrometer. ^{32}P and ^{3}H were measured simultaneously in separate channels. In a few experiments the presence of cyclic 3',5'-AMP was confirmed by paper chromatography in isopropanol, ammonium hydroxide, water (7:2:1 v/v) as described by Rabinowitz et al. (1965).

Results and Discussion. Beef thyroid homogenates accumulated cyclic 3',5'-AMP (Fig 1). TSH increased the accumulation of cyclic 3',5'-AMP, and the TSH effect was evident by 30 seconds (Fig. 1). This rapid effect suggests that the site of TSH action is either directly on adenyl cyclase or on a closely related step. The increased rate of cyclic 3',5'-AMP



Legend to Figure 1. The time course of the effect of TSH on the accumulation of cyclic 3',5'-AMP in beef thyroid homogenates. Each reaction mixture contained 0.67 mg of thyroid homogenate protein and where indicated 20 mU of TSH.

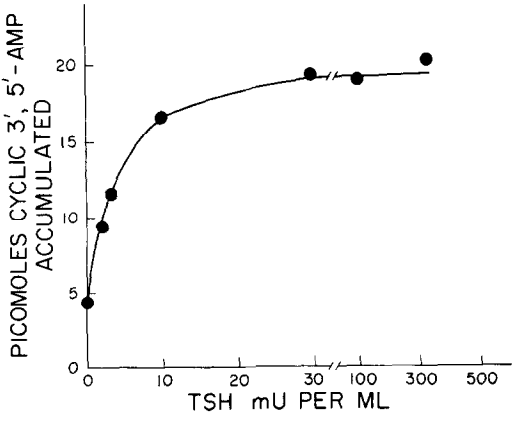


Legend to Figure 2. The accumulation of cyclic 3',5'-AMP as a function of beef thyroid homogenate concentration. Each reaction mixture was incubated for 5 minutes and contained, where indicated, 20 mU of TSH.

accumulation lasted for two minutes in the experiment shown and lasted for up to five minutes in other experiments. After incubation for ten minutes or longer the amount of cyclic 3',5'-AMP accumulated was less than at five minutes, probably because of destruction of the nucleotide by cyclic 3',5'-AMP phosphodiesterase. TSH also increased the accumulation of cyclic 3',5'-AMP in dog thyroid homogenates. The amount of nucleotide accumulated was only about 30% of that in beef.

The amount of cyclic 3',5'-AMP accumulated in the presence of TSH was proportional to the homogenate concentration (Fig. 2). The amount of cyclic 3',5'-AMP formed increased as the TSH concentration was increased over the range of 1 to 30 mU/ml (Fig. 3). Elevating the concentration of TSH to 333 mU/ml gave no greater response. The concentrations of TSH which produced 50% of the maximal response was 5mU/ml, and the apparent K_m for TSH was 3×10^{-9}M.

The increase in cyclic 3',5'-AMP was specific for TSH, since other polypeptide hormones (ACTH, chorionic gonadotrophin and growth hormone) were inactive (Table I), and the TSH preparation failed to stimulate cyclic 3',5'-AMP accumulation in adrenal homogenates where ACTH was active. Furthermore a highly purified TSH preparation (25 I.U./mg)



Legend to Figure 3. The effect of TSH concentration of the accumulation of cyclic 3',5'-AMP in beef thyroid homogenates. Each reaction mixture was incubated for 5 minutes and contained 0.45 mg of thyroid homogenate protein.

Exp't	Substance	Concentration (μg/ml)	Cyclic 3',5'-AMP accumulated (picomoles in 5 minutes)
A	--	--	4.2
	TSH	83	20.6
	ACTH	4	3.3
	Chorionic Gonadotrophin	100	3.6
B	--	--	6.2
	TSH	3.3	14.5
	Growth Hormone	10.0	6.9
	Epinephrine	100	6.1
	Acetylcholine	130	6.9

Table I. Specificity of the Effect of TSH on Adenyl Cyclase Activity. Each value is the mean of duplicates. In experiment A each sample contained 0.45 mg. of homogenate protein and in experiment B 0.57 mg.

was also active in thyroid homogenates. Epinephrine and acetylcholine were inactive. These substances have been found to increase glucose oxidation in the thyroid (Pastan et al., 1962; Pastan et al., 1961) but do not induce the formation of intracellular colloid droplets (Pastan and Wollman, 1967).

No cyclic 3',5'-AMP formation was detected in boiled homogenates or when $MgCl_2$ was omitted. When theophylline was omitted, no cyclic 3',5'-AMP accumulation was detected in the absence of TSH, but when TSH was present, a small but significant increase in cyclic 3',5'-AMP accumulation could still be detected.

The TSH mediated increase in cyclic 3',5'-AMP accumulation could be due either to an increase in the conversion of ATP to cyclic 3',5'-AMP by adenyl cyclase or to a decrease in destruction of cyclic 3',5'-AMP

HORMONE	Cyclic 3',5'-AMP Hydrolyzed (picomoles/5 minutes)
--	26.3 ± 0.8
TSH (66 mU/ml)	26.6 ± 0.3

Table II. Lack of Effect of TSH on Cyclic 3',5'-AMP Hydrolysis. Each reaction mixture contained 560 µg of thyroid homogenate. Incubation conditions were identical to those described for the adenyl cyclase assay except that ATP was omitted, and each reaction mixture contained 94.5 picomoles (1620 cpm) ^{3}H-cyclic 3',5'-AMP. The reactions were terminated by boiling for 3 minutes after the addition of 0.1 ml H_2O. Then 0.4 ml of H_2O was added, the precipitate removed by centrifugation, the supernate made up to 3.0 ml, treated twice with $ZnSO_4$ and $Ba(OH)_2$ as described under methods and the radioactivity of an aliquot of the supernate measured. Values are the mean ± SE of three samples.

by the phosphodiesterase. No effect of TSH on cyclic 3',5'-AMP destruction was found (Table 2). Therefore, TSH must act by increasing the activity of adenyl cyclase.

The first step in TSH action appears to be the binding of TSH to a superficial site, probably the plasma membrane (Pastan, Roth, and Macchia, 1966). Whether the binding site is adenyl cyclase or whether TSH influences adenyl cyclase activity indirectly is unclear. The ability to detect an effect of TSH on adenyl cyclase in homogenates provides a means to investigate this problem.

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