Evidence for the Induction of Iodide Transport in Bovine Thyroid Cells Treated with Thyroid-stimulating Hormone or Dibutyryl Cyclic Adenosine 3',5'-Monophosphate*

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SUMMARY

In isolated bovine thyroid cells, incubation with TSH (thyroid-stimulating hormone) brought about a slowly developed stimulation of iodide accumulation. This stimulation was associated with an increase in the $V_{\text{max}}$ of the system that transports iodide ion into the cell.

During the course of development of the stimulatory response, the presence of TSH was required only during the first 1 to 2 hours. After this initial period, the TSH could be removed, but full stimulation would still occur. During this same period, the stimulatory response could be completely blocked by addition of actinomycin D; whereas beyond it, addition of actinomycin D was without effect. It appears, therefore, that the TSH induced the synthesis of a specific RNA, and that this RNA accumulated sufficiently during the initial 2 hours to provide for the subsequent development of the full stimulatory response.

When cycloheximide was added along with the TSH, stimulation was completely inhibited. However, if the cycloheximide and TSH were washed out at the end of the initial 2-hour period, and the incubation of the cells continued, full stimulation of the iodide pump developed even in the absence of further additions of TSH. Evidently, the induced specific RNA was accumulated in the cycloheximide-blocked cells, and when the cycloheximide was removed, this RNA became effective in inducing the formation of a specific stimulatory protein.

Treatment of the thyroid cells with dibutyryl cyclic AMP (cyclic adenosine 3',5'-monophosphate) in place of TSH faithfully reproduced all the effects of TSH in every detail. Moreover, the cyclic AMP content of the cells was found to be acutely responsive to increases and decreases in the concentration of TSH added to the incubation media.

We conclude from the present findings that the first action of TSH is the activation of adenyl cyclase so that cyclic AMP production is augmented. Then the production of a specific RNA is induced, which in turn induces the formation of a specific stimulatory protein. The function of this protein in the stimulation of the iodide pump remains to be elucidated.

In bacteria, the induction of "permeases" involved in transmembrane transport functions has been observed and characterized in some detail (1, 2). In animal cells, such induction has not been clearly shown until now. However, it may be that this sort of mechanism will account for the stimulatory effects of aldosterone on sodium transport in the amphibian urinary bladder (3), and of vitamin D on intestinal calcium absorption (4, 5).

The stimulatory action of thyroid-stimulating hormone on thyroidal iodide transport was first shown clearly by Halmi et al. (6). They observed that a single injection of TSH into rats led, after a lag period of about 8 hours, to 50 to 100% increases in thyroidal iodide accumulation. In their report of this study, it was suggested that the latent period for this action of TSH could be attributed to the time required for the formation of new enzymes or of iodide carrier substance.

Recently we have observed the stimulatory effect of TSH added in vitro on the transport of iodide by isolated bovine thyroid cells (7-10). Iodide accumulation was observed and expressed in terms of C:M ratios, i.e. the ratios of intracellular iodide concentration to medium iodide concentration, which were determined with the aid of $^{131}$I-iodide as tracer. Following the addition of TSH to the incubating cells, a lag period of 1 to 2 hours occurred, after which C:M values gradually rose to 50 to 100% above control levels by the 6th hour after TSH addition. This action of TSH could not be duplicated by insulin, prolactin, growth hormone, or ACTH. The augmentation of C:M values could be blocked by addition of actinomycin D, puromycin, or cycloheximide (8-10), a finding which provided substantial support for the idea that the synthesis of RNA and protein moieties was involved.

* The abbreviations used are: SH, thyroid-stimulating hormone; ACTH, adrenocorticotropic; dibutyryl cyclic AMP, N'\text{6,02'-dibutyryl cyclic AMP}, cyclic AMP, cyclic adenosine 3',5'-monophosphate.

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We have also found that the TSH stimulation of iodide transport could be reproduced in every detail by 3 mM dibutyryl cyclic AMP added to the isolated cells. We have interpreted this observation to indicate that the adenylyl cyclase-cyclic AMP system participates in the mediation of this action of TSH.

In the present study, we have examined the time course of stimulation of iodide transport to identify the period during which the presence of TSH, cyclic AMP, or both is necessary, and the periods during which RNA synthesis and protein synthesis are required. These findings are consistent with the notion that TSH, mediated intracellularly by cyclic AMP, induces iodide pump activity in thyroid cells. However, many alternative mechanisms remain to be considered.

METHODS AND MATERIALS

Our technique for the tryptic dispersion and isolation of cells from bovine thyroid tissue has been described in previous reports (8,11). For the present work, this procedure was altered in that a newly designed apparatus was used for the continuous flow, tryptic dispersion of the thyroid glands. This apparatus is illustrated in Fig. 1.

The procedure for determination of 131I-C:M ratios was exactly as described previously (8). The cells were incubated in 100 volumes of Eagle’s medium (12) with 10% calf serum and 1 μCi iodide labeled with 0.1 μCi per ml of 131I. Three millimolar tapazole (1-methyl-2-mercaptoimidazole) were added so that transport of iodide occurred, but the further utilization of the iodide was blocked. The incubations were generally carried out in 25-ml Erlenmeyer flasks, under 95% O2-5% CO2 with shaking at 80 to 100 oscillations per min.

For the study of the effects of TSH and dibutyryl cyclic AMP on the V_{max} and K_{m} of unidirectional iodide influx, portions of a fresh thyroid cell preparation were incubated for 6 hours, either without added stimulators, or with 0.1 unit per ml of TSH or 3 mM dibutyryl cyclic AMP to obtain maximal stimulation. Portions of such control and stimulated cells were next equilibrated for 30 min at 37°C with media containing 100, 150, 300, and 1000 μg/100 ml concentrations of iodide. Then 131I tracer was added, and the uptake of 131I was determined at 10-, 20-, and 30-sec intervals thereafter. When the 131I uptakes (counts per min per mg of cells) were plotted against time, linear plots were obtained and the slopes of such lines provided values for the rates of 131I uptake at each of the extracellular iodide concentrations that were used. Finally, the 131I uptake rates were converted into iodide influx rates (micrograms of iodide per min per ml of cells) by dividing each 131I uptake rate by the corresponding iodide specific activity.

The treatment of the cells with actinomycin D and puromycin, and evidence that these agents inhibited RNA and protein synthesis have been described previously (13).

The determination of the cyclic AMP content of the thyroid cells was carried out according to the method of Kaneko and Field (14).

Bovine TSH (National Institutes of Health-B6, 2 units per mg) was obtained from the Endocrinology Study Section of the National Institutes of Health. Dibutyryl cyclic AMP was purchased from Boehringer Mannheim, puromycin from Nutritional Biochemicals, and cycloheximide from Sigma. Actinomycin D was generously donated by Merck Sharp and Dohme.

The reagents used for the cyclic AMP assay procedures were obtained from the following sources. Cyclic AMP was obtained from Calbiochem; 3H-cyclic AMP from Schwarz BioResearch; myokinase, pyruvate kinase, hexokinase, glucose-6-phosphate dehydrogenase, and 6-phosphogluconate dehydrogenase from Boehringer Mannheim. The phosphodiesterase used to convert cyclic AMP to AMP was prepared from rat brain by the procedure of Cheung (15).

RESULTS

Period of TSH Requirement—The critical requirement of this study was the technique for removing added TSH after various periods of incubation with the thyroid cells. For this purpose we used a tryptic treatment similar to that of Pastan, Roth, and Macchia (16) in their experiments with thyroid tissue slices. In
FIG. 3. Effects of washout on dibutyryl cyclic AMP (DBC) stimulation of I\(^{-}\) transport \((n = 6)\). Same as for Fig. 2 excepting that 3 mM dibutyryl cyclic AMP was used instead of TSH, and the dibutyryl cyclic AMP treatment was terminated by washing only without the trypsin treatment. Mean ± S.E. from six experiments.

FIG. 5. Development of I\(^{-}\) pump stimulation after prior treatment with TSH and cycloheximide \((n = 4)\). TSH \((0.1 \text{ unit per ml})\) and 0.1 mM cycloheximide were added at zero time. At the four times indicated the incubations were interrupted, and the \(^{131}\text{I}-\text{C:M}\) was determined (striped bars). Then the TSH and cycloheximide were removed by washout and treatment with 0.06% trypsin, and the cells were resuspended in fresh \(^{131}\text{I}\) media for incubation to 6 hours when final \(^{131}\text{I}-\text{C:M}\) values \((plain\ bars)\) were determined. A parallel series of control incubations without TSH were performed to provide basal \(^{131}\text{I}-\text{C:M}\) values for comparison with each of the experimental observations. The dash-outlined bars at 4 and 5 hours indicate the magnitude of TSH stimulation in samples not treated with cycloheximide. Mean ± S.E. from four experiments.

for our work, the cells were sedimented by centrifugation, washed twice with fresh Eagle's medium, incubated for 15 min at 37° with 0.06% trypsin (Worthington, once crystallized), then washed with medium containing 0.06% soybean trypsin inhibitor. Finally, the cells were resuspended in Eagle's medium containing \(^{131}\text{I}-\text{iodide}\), and incubations continued for the determination of \(^{131}\text{I}-\text{C:M}\). The trypsin concentration of 0.06% was selected after a series of tests in which solutions of TSH (0.1 unit per ml) were incubated with trypsin at 0.01 to 0.07% concentrations, and then examined for stimulatory activity on thyroid cells. The 0.06% of trypsin completely eradicated stimulatory activity without affecting basal levels of iodide transport, and protein-iodinating functions.

Accordingly, freshly isolated thyroid cells were incubated with 0.1 unit per ml of TSH for 15, 60, 150, and 270 min following which the TSH was removed, and the cells incubated further for a total time of 6 hours. Then the effect of the TSH was determined by comparing the \(^{131}\text{I}-\text{C:M}\) of TSH-treated cells with that of control cells that had been similarly incubated, but not treated with TSH. The results of five such experiments are summarized in Fig. 2, in which the effects of TSH are indicated on the ordinate in terms of the observed increases in \(^{131}\text{I}-\text{C:M}\). As shown on the bottom plot, TSH treatment for 270 min led to 50 to 60% increases in the \(^{131}\text{I}-\text{C:M}\). When the cells were exposed to TSH for only 15 min, no response could be detected. After 1 hour of TSH treatment, a barely significant increase of the \(^{131}\text{I}-\text{C:M}\) occurred at 6 hours. TSH treatment for 150 min or more elicited the full stimulatory response. It appears therefore, that the period of TSH requirement is between 1 and 2 hours. After this period, the stimulatory response is fully triggered so that the presence of TSH is no longer necessary. The interpretation of these findings is dependent upon the efficacy of the procedure for removing the TSH from the cells. The fact that this procedure completely abolished the stimulatory response in the cells that had been incubated for 15 min with TSH (Fig. 2) indicates that the trypsinic treatment was very effective.
TSH and Dibutyryl-3',5'-AMP Stimulation of Thyroidal Iodide Transport

Fig. 6. Effect of TSH (0.1 unit per ml) on cyclic AMP (cAMP) in bovine thyroid cells. Thyroid cells were incubated at 37° in Eagle's medium containing 10 mM aminophylline, without or with TSH. At the times shown, the cells were sedimented and homogenized in 0.1 N HCl to extract the cyclic AMP.

Fig. 7. Effect of TSH addition and withdrawal on cyclic AMP (cAMP) in thyroid cells. A, thyroid cells were incubated for 20 min in Eagle's medium with 10 mM aminophylline without or with 0.1 unit per ml of TSH, then sedimented and extracted for cyclic AMP. B, same as in A, but then washed and incubated 15 min with 0.06% trypsin before resedimentation and extraction for cyclic AMP. C, same as in B, but then resuspended in fresh medium with 0.1 unit per ml of TSH and incubated for 5 min before resedimentation and extraction for cyclic AMP. Mean ± SE from three experiments.

Indeed. Further evidence is presented below in the description of the effects of TSH on intracellular cyclic AMP levels.

Period of Response to Dibutyryl Cyclic AMP—In this study, thyroid cells were incubated without and with 3 mM dibutyryl cyclic AMP for periods of 15, 60, 150, and 270 min. Then they were sedimented and washed three times with fresh media to remove the dibutyryl cyclic AMP, resuspended in media with 1311-iodide, and incubated for a total of 6 hours. The results of this study (Fig. 5) show that the action of dibutyryl cyclic AMP, like that of TSH, was completed within the first 1 to 2 hours of treatment. After this time, full stimulation of iodide accumulation occurred even in the absence of dibutyryl cyclic AMP.

Again the efficacy of the washout procedure for removing the added dibutyryl cyclic AMP is evidenced by the failure to observe stimulation of 1311 accumulation in the 15-min dibutyryl cyclic AMP-treated cells.

Actinomycin-sensitive Period—Thyroid cells were incubated without additions and also with 0.1 unit per ml of TSH or with 3 mM dibutyryl cyclic AMP. Actinomycin D, 5 μg per ml, was added at zero time, 40, 100, or 150 min after the beginning of incubation. After 6 hours of incubation, 1311-C/M values were determined. As shown in Fig. 4, actinomycin D added during the first 40 min inhibited the stimulation of iodide accumulation by TSH. When the actinomycin D was added after 100 min of treatment with TSH, no inhibition could be detected. Similar results were obtained when dibutyryl cyclic AMP was used in place of TSH. We conclude, therefore, that the induction of new RNA synthesis was completed within 100 min of TSH or dibutyryl cyclic AMP treatment.

Accumulation of Induced RNA—We have reported previously that puromycin (8) and cycloheximide (9, 10) block the stimulation of iodide transport elicited by TSH and dibutyryl cyclic AMP. If the cycloheximide inhibits only the synthesis of protein and does not interfere with RNA production, then it is possible that newly formed RNA will accumulate in the cells when they are treated with TSH or dibutyryl cyclic AMP in the presence of cycloheximide. And if this accumulated RNA is still capable of directing new enzyme synthesis, it should be possible to get the stimulatory process to proceed to completion by washing out the cycloheximide and continuing the incubation. Indeed, if our conception of the stimulatory process is correct, the stimulation should proceed to completion and provide maximum stimulation even after the TSH and dibutyryl cyclic AMP are also removed. The results of such experiments are illustrated...
in Fig. 5. The uppermost plot shows that 1 hour of TSH treatment in the presence of cycloheximide did not significantly alter the \(^{131}I\text{-C}:M\) even when incubations were continued for 4 hours after washing out the TSH and cycloheximide. Evidently, little RNA accumulated during the 1-hour treatment with TSH. When the TSH and cycloheximide treatment was extended to 2 hours, there was no change in the \(^{131}I\text{-C}:M\) at the time that the TSH and cycloheximide treatment was terminated. However, continued incubation for 4 more hours led to near maximal increase in the \(^{131}I\text{-C}:M\). When the cells were incubated with TSH and cycloheximide for 4 to 5 hours, again the \(^{131}I\text{-C}:M\) at the end of these periods was not above basal levels. As indicated by the dashed-outlined bars, considerable increase in the \(^{131}I\text{-C}:M\) would have occurred at these times had the cycloheximide not been present. When these cells were washed free of TSH and cycloheximide, continued incubation led to prompt increases in the \(^{131}I\text{-C}:M\).

A series of similar experiments was also done with dibutyryl cyclic AMP in place of TSH. Again, it was found that a 1-hour stimulation period did not provide sufficient RNA to accumulate for a stimulatory response to be elicited, whereas 2 or more hours of dibutyryl cyclic AMP treatment were adequate to produce a prompt maximal response even after the removal of the dibutyryl cyclic AMP.

**Regulatory Action of TSH on Cyclic AMP Production**—As illustrated by the results summarized in Fig. 6, addition of TSH to the thyroid cells increased the cyclic AMP content of the cells by 85% within 1 minute and maintained the elevated values for as long as 100 minutes. Other hormones, such as vasopressin (50 \(\mu\text{g per ml})\), insulin (1.6 units per ml), glucagon (80 \(\mu\text{g per ml})\), ACTH (5 units per ml), and epinephrine (60 \(\mu\text{g per ml})\), which influence adenylyl cyclase activities in certain other tissues (17), did not detectably alter the cyclic AMP content of the thyroid cells.

The results presented in Fig. 7 show that the TSH-evoked elevations of cyclic AMP content could be returned within 15 minutes to basal levels when the TSH was removed by the washout and tryptic treatment, and then promptly raised again when TSH was added a second time. The latter finding indicates that the tryptic treatment did not damage the cells sufficiently to prevent a subsequent response to a second addition of TSH.

In five experiments, it was observed that in cells incubated for 1 or 2 hours with TSH, the elevated cyclic AMP levels could be returned promptly to basal values by the tryptic treatment. Evidently the TSH engaged in the stimulation of adenylyl cyclase remains on the surface of the cells even after such prolonged incubations.

**Effects of TSH and Dibutyryl Cyclic AMP on Iodide Pump**—The experimental findings described so far indicate that in the TSH- and dibutyryl cyclic AMP-treated cells, a protein is induced, and the production of this protein leads to increased iodide accumulation. In order to characterize further the mechanism responsible for these alterations in iodide transport, we undertook a more intensive examination of the iodide pump which mediates the iodide influx process. The experimental approach, which is described under "Methods and Materials," was adapted from the Michaelis-Menten formulation for the analysis of enzyme reaction kinetics. The data from a typical experiment, plotted after the mode of Lineweaver and Burk, are shown in Fig. 8, and the results from four additional experiments are summarized in Table I. These results show that the \(K_m\) for unidirectional iodide influx into the cells was not changed in the TSH- and dibutyryl cyclic AMP-stimulated cells. Instead, the \(V_{\text{max}}\) was increased by 50 to 80% above basal levels.

**DISCUSSION**

It is evident from this study that cyclic AMP production by bovine thyroid cells is sharply increased by added TSH and promptly returned to basal levels after removal of the TSH by treatment with trypsin. These findings are in good agreement with the view that the first step in TSH action is the binding of the hormone to receptor sites on cell surface (16), followed by activation of membrane-bound adenylyl cyclase (20, 21), and the resultant increase in the production of cyclic AMP (22, 23). Evidently such an increase in intracellular availability of cyclic AMP then functions in cascade fashion to bring about all the divergent metabolic and morphological changes which occur in response to TSH treatment. Presumably, it is by artificially increasing cyclic AMP supply that added dibutyryl cyclic AMP can reproduce virtually all of the actions of TSH (8, 24-26). Thus abundant, although not yet conclusive, evidence has accumulated that the adenyl cyclase-cyclic AMP system functions in the mediation of TSH action in the manner suggested by Robison, Butcher, and Sutherland (17).
In the sequence of reactions triggered by TSH and leading to the stimulation of iodide transport, the third step appears to be the production of RNA. As gauged by the period of sensitivity to actinomycin D, this step requires 1 to 2 hours in order to produce enough RNA for maximal stimulation of the iodide pump. Since the continued presence of either TSH or dibutyryl cyclic AMP is essential during this same 1- to 2-hour period, we conclude that the RNA production step is dependent upon a continuous, TSH-stimulated supply of cyclic AMP. Afterward, the fourth step, involving the translational (cycloheximide-sensitive) events, will proceed maximally even in the absence of TSH.

We have previously adduced experimental evidence that TSH stimulates protein synthesis in thyroid tissue slices and isolated thyroid cells (13). It seems likely that a portion of this enhanced protein synthesis is concerned with increasing thyroglobulin production (9, 10). However, some of it may be involved in the stimulation of iodide transport and we are in the process of searching for this specific protein.

In the dispersed bovine thyroid cells, TSH does not reproducibly alter nucleic acid synthesis (13), in a detectable fashion. We conclude tentatively that the TSH-induced, specific RNA concerned with stimulating the iodide pump is produced only in small quantities and could not be detected against the background of ongoing RNA production.

The TSH-induced protein has not been isolated or characterized, so that its exact function in the stimulation of iodide transport remains to be defined. Our finding that the V_max for the iodide pump was increased in the stimulated cells suggests that the TSH-induced protein has a function that is rate-limiting for iodide pump activity.

REFERENCES

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