Biphasic Release of Thyrotropin in Response to Thyrotropin-Releasing Hormon (TRH) from Rat Anterior Pituitary Cells *in Vitro*: Possible Dependence on Protein Synthesis

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Abstract

Mechanisms related to the biphasic release of TSH were studied using primary cultured cells of the rat anterior pituitary gland on micro-carrier beads in a superfusion system. Release of TSH in response to continuous exposure to TRH exhibited a biphasic pattern; the first phase was characterized by a rapid, transient and high-rate release (phase I) and the second phase by a chronic and low-rate release (phase II). The shift of the release from phase I to phase II occurred by treatment with TRH at concentrations from submaximal to maximal. When the Ca^{2+} concentration in the medium was decreased, the phase I release was partially inhibited, while the phase II release was completely inhibited, suggesting a difference between the mechanisms in phase I and phase II release. The phase I release was not suppressed by cycloheximide. This protein synthesis-independent release of phase I seemed to be linked to the intracellular releasable pool of TSH. The phase II release was suppressed by the presence of a protein synthesis inhibitor. After the phase II release was suppressed by cycloheximide, the magnitude of phase I release in response to re-exposure to TRH markedly decreased. The decreased phase I release in response to TRH was observed with the cells which were previously stimulated by high K^+ instead of TRH, suggesting that the decrease in the response of phase I reflects the depletion of a releasable pool of TSH rather than homologous desensitization of thyrotrophs with TRH. These results suggest that the phase I release of TSH depends on a portion of the previously prepared-releasable pool while phase II release depends on previously prepared plus newly prepared pools of TSH. Replenishment of the releasable TSH pool was considered to involve protein synthesis.

During constant stimulation by an appropriate agent, there is a biphasic response of several protein hormones including LH (Tixier-Vidal and Gourdg, 1981), PRL and TSH (Morin *et al.*, 1975; Bremner *et al.*, 1977; Schrey *et al.*, 1977; Connors *et al.*,

1981) *in vivo and in vitro*. It seems, therefore, that there might be a common release mechanism for all of these hormones. Bremner *et al.* (1977) reported that constant intravenous infusions of TRH resulted in a TSH release in two phases in healthy humans and suggested that these two phases of release may reflect the existence of two

pools of TSH in the cells. Schrey *et al.* (1977) and Connors *et al.* (1981) demonstrated that continuous exposure to TRH resulted in a biphasic release of TSH *in vitro* using dispersed cells of the rat anterior pituitary. We also demonstrated that TRH as well as its analog (DN-1417) caused biphasic release of TSH from superfused pituitary cells (Shiota *et al.*, 1984). Since the profile of the biphasic release caused by a maximally effective concentration of TRH was different from that by high K^+ (60 mM) solution, there seems to be a specific action of TRH related to the biphasic release (Shiota *et al.*, 1984). Though protein synthesis is theoretically expected to play an important role in the second-phase release, as shown in the case of LH (Pickering and Fink, 1976; Kercret and Duval, 1978; Liu and Jackson, 1978; Bourne and Baldwin, 1980), the relation of the first and the second release of TSH in connection with protein synthesis is poorly understood.

Though a superfusion technique appears suitable for investigating the transient phenomena of hormone release, the acquisition of pertinent data is difficult due to considerable inter-experimental variation when a one-channel superfusion system is used. We attempted to characterize the biphasic release of TSH in a specially designed multi-channel superfusion system in which data can be compared within an experiment and to clarify the dependence of this on protein synthesis. The effect of extracellular Ca^{2+} was also studied.

Materials and Methods

Animals

Male Sprague-Dawley rats (ages 9–12 weeks, CLEA Japan Inc.) were maintained in a controlled environment at $24 \pm 1^\circ C$ with a 14 h light–10 h dark cycle (lights on at 0730 h) and had free access to water and standard laboratory chow.

Reagents

TRH was synthesized in the Chemistry Laboratories of the Takeda Central Research Division (Fukuda *et al.*, 1980). Other materials were obtained from the following sources: cycloheximide, BSA (fraction V) and ethyleneglycol-bis-(β -aminoethyl ether) N, N, N', N'-tetraacetic acid (EGTA) from Wako Pure Chemical Industries, LTD. (Japan); DNA (type I from calf thymus), and gentamicin sulfate from Sigma Chemical Co. (St. Louis, Mo); pancreatin, Hepes, DNase, Dulbecco's Modified Eagle Medium, penicillin and streptomycin from Grand Island Biological Co. (Grand Island, NY); collagenase (type II) from Millipore Corporation (Freehold NJ); and CytodexTM1 from Pharmacia Fine Chemicals (Sweden).

Primary culture of anterior pituitary cells and superfusion

The primary culture of the cells and superfusion were performed by a modification of methods reported elsewhere (Shiota *et al.*, 1984; Shiota and Wiest, 1979; Smith and Vale, 1980). Briefly, rat anterior pituitary glands were quartered and washed with Hepes buffer (137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4 , 25 mM Hepes and 50 $\mu g/ml$ gentamicin sulfate, pH 7.3). The tissue was dispersed by incubation first with a buffer containing 0.4 % collagenase, 0.4 % BSA, 10 $\mu g/ml$ DNase and 0.2 % glucose, and then with 10 % pancreatin. Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % fetal bovine serum, 20 mM Hepes, 50 U/ml penicillin and 50 $\mu g/ml$ streptomycin was added to bring the concentration to 3×10^5 cells/ml. CytodexTM1, swollen in Hepes buffer (0.1 g/2.5 ml), was mixed with 100 ml lots of the cell suspension and incubated for 4–5 days under a saturated atmosphere of 5 % CO_2 –95 % air in a CO_2 incubator at $37^\circ C$.

Cells attached to the CytodexTM1 beads (0.9 – 1.4×10^7 cells/column) were introduced in a 0.7×4 cm polypropylene/glass column (Bio-Rad Laboratories, Richmond, CA), and the columns were placed in a $37^\circ C$ water bath. The superfusion medium consisted of DMEM (pH 7.3) containing 1.85 g $NaHCO_3$ /liter supplemented with 20 mM Hepes, without serum and antibiotics. In certain experiments, Krebs Improved Ringer I bicarbonate solution (McKenzie and Dawson, 1969) was used as a superfusion medium (KIRI). A high K^+ (60 mM) solution was prepared by substituting KCl for NaCl to the extent necessary. A

low Ca^{2+} medium was prepared by simply omitting CaCl_2 , with or without adding EGTA (16 μM). The Microperpex® peristaltic pumps (LKB-Produkter, Sweden) maintained the flow of the medium through the cell column at a constant rate of 0.25 ml/min. The medium was allowed to flow down the side wall of the column. A lag period of 13.5 ± 0.6 min ($n=4$) was needed for the peptide to reach the fraction collector. Two to four columns were run in parallel and one served as the control in every experiment. The cell columns were allowed to adjust to the superfusion environment for 120 min before sample collection. The effluent was collected every 10 min. The samples were stored at -20°C until assay. At the end of the superfusion, cells were recovered and suspended in 1 ml saline and stored at -20°C until assayed for TSH and DNA. TRH and cycloheximide were dissolved in the medium to give the final concentration shown under Results. TRH in the superfusion medium proved to be stable under these experimental conditions, because 96 % of the initial concentration of TRH was recovered 6 h after peptide was added to the medium at room temperature (Shiota *et al.*, 1984).

Assays

An aliquot of the cell suspension was homogenized in 2 ml 50 mM Tris-HCl buffer containing 12.5 mM sodium borate and 5 mM EDTA, pH 8.9 (Jahn *et al.*, 1982) and sonicated with a Sonic Dismembrator (ARTEK System Corp., Farmingdale, NY) at dial 60 for 20 sec. After centrifugation (3,000 rpm, 20 min), an aliquot of the supernatant was used for the determination of cellular TSH content. The DNA content in the cells was measured by a modification of the method of Burton (1956), using type I calf thymus DNA as the standard.

The amount of TSH was measured in duplicate by the double antibody radioimmunoassay using a kit provided by the NIAMDD Rat Pituitary Hormone Distribution Program. Iodination of TSH with ^{125}I was performed by the chloramine-T method. All samples of the medium were assayed in duplicate using 200 μl undiluted samples. The results were expressed in terms of NIAMDD-rat TSH-RP-1 standard. The intra-assay coefficient of variation was 5.7 % ($n=5$) and the inter-assay coefficient of variation was 16.6 % ($n=9$).

Results

Each result shown is a representative experiment out of two or three similar experiments with similar results.

Effects of continuous exposure to fixed or various doses of TRH on TSH release.

Four columns were run in parallel, and the TSH responses to fixed or various doses of TRH were examined (Fig. 1). DNA content was 104.4 ± 2.6 $\mu\text{g}/\text{column}$ ($n=4$) in the experiment. The intra-experiment coefficient of variation of the DNA content was less than 5 % throughout. The cells were first exposed to TRH (100 nM) for 100 min and the responses were nearly identical for all columns. During the continuous exposure to TRH, a high rate of TSH release was initially observed within 6.5 min after the addition of TRH (the time-lag, 13.5 min was deducted), for about 40 min (phase I); thereafter the hormone release decreased rapidly to a constant lower rate of secretion (phase II) even with continuous exposure to TRH. Phase II release lasted as long as the cells were exposed to TRH; when TRH was withdrawn, the TSH release decreased to the basal level.

Sixty min after the end of the first exposure to TRH, the cells were re-exposed for 60 min to varying concentrations of TRH (0, 1, 5 and 100 nM) and the dose-dependent increase in phase I and phase II release of TSH was obtained, indicating that the rapid decrease in phase I release or shift to phase II release was not due to nonspecific damage of the cells as there was a re-occurrence of the response. The maximally effective concentration of TRH was 100 nM, since 1 μM of TRH produced no further increase in the hormone release (data not shown).

Effects of continuous exposure to TRH in low Ca^{2+} medium

To evaluate the extracellular Ca^{2+} de-

Fig. 1. TSH release in response to continuous exposure to TRH. The cells were first exposed to TRH (100 nM for 100 min) followed by various doses (0, 1, 5, 100 nM for 60 min) at 60 min interval. Four columns were run in parallel with a flow rate of 0.25 ml/min and the effluent was collected every 10 min. Time scales indicate the period after the start of either sample collection or TRH stimulation.

dependency of the biphasic release of TSH, the cells were exposed to TRH (100 nM) for 180 min in a low Ca^{2+} medium (Fig. 2A). Exposure to the low Ca^{2+} medium was initiated 40 min before the TRH stimulation. Basal release of TSH was not reduced in low Ca^{2+} medium. During the exposure to TRH, there was a slight increase in release in the period corresponding to the phase I, but the phase II release was completely inhibited under conditions of low Ca^{2+} . EGTA (16 μM), a chelating agent, was added simultaneously with TRH in the low Ca^{2+} medium (Fig. 2B). A slight increase in TSH in the phase I period was still apparent.

Effects of a protein synthesis inhibitor on TSH release

In order to further characterize the biphasic release of TSH, the dependency of each phase release on protein synthesis was studied. The effect of the protein synthesis inhibitor, cycloheximide (CX), on the release of TSH was then examined (Fig. 3). The cells were exposed to TRH (100 nM) for 180 min in the presence or absence of 7 μM CX, a dose that inhibited [methyl ^3H]

methionine incorporation of primary culture of pituitary cells into trichloroacetic acid precipitable material by about 80 % in static incubation for 2 or 4 hr. When the cells were exposed simultaneously to CX and TRH, there was no inhibitory effect on phase I release of TSH, while phase II release was reduced. When exposure to CX (7 μM) was started 120 min before the continuous exposure to TRH, the basal and the phase I releases of TSH were not reduced but the phase II release was again suppressed (data not shown), confirming that the phase I release was refractory to the protein-synthesis inhibitor and the result was not due to a CX action time lag. The dependency on protein synthesis appeared at a fixed time of TRH stimulation—about 80 min after the initiation of TRH stimulation—regardless of the duration of the CX pretreatment. The net amounts of TSH released during TRH exposure were about 6 % (control) and 5 % (in the presence of CX) of the original immunoreactive TSH content in the cells. At the end of the experiment, a 293 μg /column and 273 μg /column of immunoreactive TSH remained in the control

Fig. 2. TSH release in response to continuous exposure to TRH (100 nM for 180 min) in normal KIRI or in low Ca^{2+} KIRI with or without EGTA. A, exposure to the low Ca^{2+} medium was started 40 min before the TRH exposure; B, exposure to the low Ca^{2+} medium was started 40 min before the exposure to TRH plus EGTA (16 μM).

and the CX treated cells, respectively. Thus, the phase II but not the phase I release of TSH in response to initial exposure to TRH seems to depend on protein synthesis.

TSH release induced by pulse exposure to TRH and the effects of CX

To examine the necessity of TRH stimulation for the appearance of the phase II release, the length of TRH exposure was

Fig. 3. TSH release in response to continuous exposure to TRH (100 nM for 180 min) in the presence or absence of cycloheximide (7 μM). The cycloheximide was present in the medium from the start of exposure to TRH.

shortened to 20 min (Fig. 4A). There was an initial rapid and transient increase but the release corresponding to phase II was not observed by this pulse exposure, indicating that continuous exposure to TRH was necessary for the biphasic release of TSH.

A pulse exposure to TRH (100 nM, 20 min) was then repeated three times at 30 min intervals in the presence or absence of CX (7 μM) and the results were compared with those for continuous exposure to TRH (Fig. 4B). Though TSH releases induced by the second and third exposures to TRH were smaller than the release induced by

Fig. 4. A, TSH release in response to a pulse (20 min) or continuous (180 min) exposure to TRH (100 nM). B, TSH release in response to three-pulse exposure (20 min) to TRH (100 nM) at 30 min intervals in the presence or absence of cycloheximide (7 μ M). Cycloheximide was continuously present from the first exposure to TRH. TSH release by three-pulse exposure was also compared with that by continuous exposure to TRH (100 nM for 120 min).

the first exposure, they were higher than the phase II release induced by the continuous stimulation and therefore more likely to be the phase I release (Fig. 4B). In the presence of CX, the repeated TRH pulses again induced 3 phase I-like releases, though there was a tendency toward a gradual decrease in the magnitude of the third release (Fig. 4B).

Fig. 5. TSH release in response to two continuous exposures to TRH in the presence or absence of cycloheximide (7 μ M). A, continuous exposure to TRH (100 nM for 120 min) was repeated twice at 60 min interval in the presence or absence of cycloheximide; B, TRH stimulation (100 nM for 120 min) was started 180 min after the initiation of cycloheximide exposure. The response with two continuous exposures to TRH in the absence of cycloheximide is included for comparison.

Effects of a protein synthesis inhibitor on TSH release induced by repeated prolonged stimulation

In the cells in which the phase II release was suppressed by treatment with a protein synthesis inhibitor, the response to the following TRH stimulation was examined. Continuous exposure to TRH (100 nM, 120 min) was repeated twice at 60 min intervals in the presence or absence of CX (Fig. 5).

Fig. 6. TSH release in response to high K⁺ KIRI (60 mM KCl for 120 min) followed by TRH (100 nM for 120 min) exposure at 60 min interval in the presence or absence of cycloheximide (7 μ M).

During the first exposure to TRH, the phase I release of TSH was not reduced in the presence of CX (7 μ M), but phase II secretion was reduced (Fig. 5A). On the second exposure to TRH, the phase I as well as the phase II release of TSH was reduced in the presence of CX. When the first exposure to TRH was omitted and the TRH stimulation was started 180 min after the initiation of CX exposure, the phase I release of TSH was not suppressed but the phase II was suppressed during exposure to TRH (Fig. 5B). Therefore, the reduced response of phase I release of TSH in response to the second exposure to TRH was not due to nonspecific damage of the cells by prolonged treatment with CX.

To determine whether the attenuated response to the second TRH stimulation may be due to a specific action of the previous exposure to TRH or simply the depletion of TSH in the cells, the first stimulation was performed by prolonged exposure to high K⁺ solution instead of TRH. The cells were first exposed to high K⁺ (60 mM) for 120 min and then exposed to TRH (100 nM) in KIRI for 120 min either in the presence or absence of CX (Fig. 6). Continuous exposure

to the high K⁺ medium resulted in a marked increase in TSH release. The phase I release of TSH induced by high K⁺ was not affected by the presence of CX (7 μ M), however, the later release corresponding to the phase II release was reduced. As in the results in Fig. 5A, both phase I and phase II releases induced by the TRH exposure following high K⁺ stimulation were considerably reduced in the presence of CX.

Discussion

The biphasic profile of TSH release was reproducible in the present study, thus confirming and extending previous studies (Schrey *et al.*, 1977; Connors *et al.*, 1981; Shiota *et al.*, 1984).

Generally, only a small percentage of the TSH content in the cells was released and large amounts of hormone remained in the cells at the end of superfusion with TRH. Immunoreactive TSH in the cells may consist of heterogeneous forms of hormone (Yora, 1979) and/or those at various stages of synthesis; there seems to be a limited pool of releasable TSH. The phase I release of

TSH dropped sharply without staying long at the peak level and the phase II release followed. This shift from phase I to phase II was observed not only with a maximum dose (100 nM) but also with a submaximum dose (5 nM) of TRH, and the amount of hormone released during phase I period by the small dose of TRH was smaller than that released by the maximum dose. Thus, the amount of hormone released during phase I does not seem to be a factor regulating the shift. Exposure to a high K^+ concentration produced a higher peak of TSH compared with that seen with a maximum stimulation dose of TRH (Shiota *et al.*, 1984), and intermittent stimulation of TRH caused phase I-like releases that were higher than the phase II release even in the presence of CX. These results indicated that the potential amount of releasable pool of hormone is usually greater than that released during the phase I, and also suggested that the shift from phase I release to phase II is caused through a mechanism other than exhaustion of the previously prepared-releasable pool.

The importance of extracellular Ca^{2+} (Schrey *et al.*, 1978; Tashjian *et al.*, 1978; Thorner *et al.*, 1980; Geras *et al.*, 1982) and intracellular Ca^{2+} mobilization (Schrey *et al.*, 1978; Gershengorn, 1980; Dust and Martin, 1982; Geras and Gershengorn, 1982; Geras *et al.*, 1982) have been reported in the release mechanism of pituitary hormones. It has not been reported, however, whether the importance of Ca^{2+} resides in the phase I or the phase II release of TSH. We found that the phase II release of TSH completely depends on extracellular Ca^{2+} , while the phase I release seems to consist of two components, extracellular Ca^{2+} -dependent and -independent. This partial dependence of the phase I release seems to be specific for TRH, because high K^+ solution did not produce any increase in TSH in the low Ca^{2+} solution, as shown in our previous study using the same superfusion system

(Shiota *et al.*, 1984). Though the possibility that a very low concentration of Ca^{2+} remains in the superfusion-column cannot be excluded during the early period of superfusion, these results suggest the existence of different mechanisms in the phase I and phase II releases. The shift in TSH release from phase I to phase II may relate to the change in Ca^{2+} action mechanism.

This is the first demonstration that the phase II release of TSH involves protein synthesis. Though the protein synthesis-dependent release in LH has been reported (Pickering and Fink, 1976; Kercret and Duval, 1978; Liu and Jackson, 1978; Bourne and Baldwin, 1980), the significance of the dependency in the dynamics of the hormone secretion is still unknown. Since the shift from phase I to phase II release occurs before complete depletion of a previously prepared-releasable pool, it is possible that the phase II release in an early period is also consuming the releasable pool that had been prepared. Because both the release of phase I and the initial part of phase II were independent of protein synthesis, the release from the previously prepared-releasable pool of TSH seems to be independent of *de novo* protein synthesis. Thyrotrophs can release TSH without *de novo* protein synthesis when TRH stimulation is given intermittently; the phenomenon was only observed before complete depletion of the releasable pool. The decrease in phase II release by CX resulted in attenuation of the phase I release induced by the following TRH stimulation, suggesting that the previously prepared pool of TSH is consumed during the previous prolonged TRH stimulation but not restored in the presence of CX. Though homologous desensitization by prolonged exposure to hypothalamic peptides has been proposed (Hinkle and Tashjian, 1975; Gershengorn, 1978; Bourne and Baldwin, 1980; Badger *et al.*, 1983), the attenuated response does not seem to depend on the down regulation by TRH because a similar result was obtained

when TSH release was first stimulated by K^+ instead of TRH. Furthermore, the period of the first exposure (120 min) to TRH seems to be too short to cause a significant reduction in the receptor in the present study. Long term treatment with TRH was required to cause a significant reduction in the receptor in *in vitro* experiments performed by others (Hinkle and Tashjian, 1975; Gershengorn, 1978). For example, a decrease to 75 % of the control was observed after 48 h exposure to TRH in GH_3 cells (Hinkle and Tashjian, 1975) and a decrease to 70 % at 24 h exposure in mouse pituitary thyrotropic tumor cells (Gershengorn, 1978). In addition, our results showed that CX treatment decreases the response; this is in contrast to the report by Hinkle and Tashjian (1975) who demonstrated that the receptor loss was reversed by the treatment with CX. Therefore, these observations strongly suggested that the attenuated response of the phase I release in the phase II blocked cells by CX depends on the depletion of a releasable pool of TSH but not on the down-regulation by TRH. The releasable pool must be replenished to continue the release after depletion of the pool, and the mechanism of the replenishment seems to involve protein synthesis.

In relation to the replenishment mechanism, *de novo* synthesis of TSH might be important, since TRH reportedly stimulates the synthesis as well as the release of TSH (Wilber, 1971; Vale *et al.*, 1972; Marshall *et al.*, 1981). In the incubation study of rat hemipituitaries with $[^{14}C]$ alanine and $[^{14}C]$ glucosamine, Wilber (1971) found that TRH stimulated the release of newly synthesized TSH and that CX inhibited the synthesis. From the studies of GH release using GH_3 cells, Stachura (1982) proposed that there is a hormone pool which is outside the direct path of the intracellular hormone flow from synthesis to release, and once discharged by the stimulation with K^+ or dibutyryl cyclic AMP, the hormone compo-

nent is rapidly reconstituted with synthesized hormone. Such a mechanism as in the case of GH release from GH_3 cells may be involved in TSH release. Another possibility is that the synthesis of protein is involved in the mechanism of the secretory process including, processing, glycosylation (Wilber, 1971; Weintraub *et al.*, 1980; Chinn and Habener, 1981; Weintraub *et al.*, 1983), packaging hormones within secretory granules and migration of the granule toward the plasma membrane as shown in morphological studies (Wilber and Spicer, 1980).

In conclusion, concerning the biphasic release of TSH in response to continuous exposure to TRH, the results described above suggested that 1) the phase I release involves both extracellular Ca^{2+} -dependent and -independent mechanisms, and the phase II release was extracellular Ca^{2+} -dependent, 2) the phase I release shifts to the phase II release through a mechanism other than depletion of the releasable hormone, 3) the phase I release depends on a portion of a previously prepared-releasable pool and the phase II release depends on both previously prepared and newly prepared pool of TSH and, 4) the replenishment process of releasable TSH pool involves *de novo* protein synthesis.

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