Factors Affecting Thyrotropin Secretion in Superfused Rat Anterior Pituitary Cells

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        The use of static in vitro pituitary cultures has been indispen-
sible for examining the regulation of anterior pituitary hormone secretion.
While the static cultures have shown the effects of various chemical
stimulators of hormone secretion, the dynamics of such secretion has not
been assessed before in vitro. The in vitro superfusion method, which

partially simulates in vivo physiologic conditions, allows for the observation of the dynamics of pituitary hormone secretion.

Enzymatically dispersed anterior pituitary cells were superfused with Krebs-Ringer Bicarbonate buffer (KRB, 0.5 mls/min) and various stimulators in order to assess the dynamics (rapidity, duration and magnitude) of thyrotropin (TSH) secretion. Thyrotropin-releasing hormone (TRH), diburtyry cyclic AMP, stalk median eminence extract (SME), potassium ion, somatostatin and TSH were used to stimulate the cells. The superfusate was collected in 1,0 ml fractions and TSH concentration determined by radio-immunoassay.

Dispersed cells maintain a stable baseline secretion of TSH when superfused with KRB and respond rapidly to two-minute pulses of TRH, SME and potassium. Dibutyryl cyclic AMP elicited no TSH response when pulsed for two or ten minutes. Somatostatin inhibits the TRH-mediated secretion of TSH while not having an inhibitory effect on baseline, potassium-mediated and SME-mediated TSH secretion. Finally, TSH is shown to inhibit TRH-mediated TSH secretion when TRH is presented to the cells in the presence of 0.5 or 1.0 ug TSH/ml.

FACTORS AFFECTING THYROTROPIN SECRETION

IN SUPERFUSED RAT ANTERIOR PITUITARY CELLS

by

FREDERICK SCOTT BARTLOW

A thesis submitted in partial fulfillment of the

requirements for the degree of

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TO THE OFFICE OF GRADUATE STUDIES AND RESEARCH:

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TABLE OF CONTENTS

PAGE

CHAPTER

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

The physiological function of the hypothalamus, hypophysis and the hypothalamic-hypophyseal portal vascular system has been the object of much investigation in many in vivo and in vitro systems for the past several decades. Recently, a new in vitro model system has been developed which offers a new approach to neuroendocrine research. The model system utilizes a continuous media flow through a chamber containing pituitary cells. This superfusion system partially simulates conditions found in vivo. Analysis of superfusate fractions collected allows investigators to observe dynamic secretory responses to stimulation, responses not observed before in static pituitary cell cultures. Several endocrine systems have been investigated using the superfusion technique, but only recently has the technique been used for the study of thyrotropin (TSH) release (Connors and Hedge, 1980; Connors et al., 1979; Smith and Vale, 1980).

This study utilizes the superfusion system to observe dynamic TSH responses when cells are stimulated with substances believed to inhibit or elicit TSH secretion. These responses are compared with results from static in vitro and superfusion experiments of other investigators. Also investigated are TSH shortloop feedback and the effects of repeated stimulation of pituitary cells on the dynamics of TSH secretion.

CHAPTER II

LITERATURE REVIEW

Anterior pituitary hormone secretion is under the control of the hypothalamus by way of the hypothalmic-hypophyseal portal system (Martin et al., 1977). Most early physiological and biochemical studies utilized in vivo experiments in which isolation of individual control mechanisms is exceedingly difficult. But the approach to neuroendocrine studies broadened in the mid-1950's to include in vitro methods, providing a method to isolate some of the experimental variables. In spite of widely varying conditions of cultures and methods of analysis used, one major conclusion was drawn: cultured anterior pituitary tissue can maintain hormone secretion for a limited period of time (Gaillard and Schaberg, 1965; Tixier-Vidal, 1975). The in vitro studies offered an improved method for examining the putative hypothalamic releasing factors which had been indicated in earlier in vivo studies (Green and Harris, 1947; Harris and Jacobsen, 1952).

The in vitro preparations of pituitary tissue allowed investigators to study pituitary hormone secretion while avoiding problems associated with the in vivo studies. Some of the problems encountered in vivo included the physical inaccessability of the pituitary gland, undetectable plasma concentrations of pituitary hormones, cellular heterogeniety of the pituitary gland, and unknown, uncontrollable variables attributed to the systemic environment (Farquar et al., 1975; Tixier-Vidal, 1975). Pituitary cultures circumvented some of the problems of accessability,

allowed the collection of hormone secretions from the culture medium, and eliminated the unknown variables of the systemic environment (Tixier-Vidal, 1975). The problem of cellular heterogeniety of the gland was dealt with at a later time by Hymer et al. (1975).

The value of in vitro pituitary culture was realized after the initial in vitro experiments provided evidence of a specific releasing factor unique to the hypothalamus. In 1955, two teams of investigators, working independently, reported the release of adrenocorticotropin from cultured whole pituitaries. Other important discoveries were made through the culture of whole pituitaries or hemi-pituitary glands for the next fifteen years (Gaillard and Schaberg, 1965) including the identification of the six hormones secreted by the anterior pituitary gland (Table I).

The in vitro culture methods permitted valuable changes in the experimental design for pituitary studies. A single gland could be divided in order to use one half of the gland for experimental treatments, the other as an experimental control. Hemi- or quartered pituitaries respond well to releasing factors found in vivo. It was noted, however, that even small molecular weight molecules exhibited poor penetration of pieces of tissue (Farquar et al., 1975). When pituitaries were divided into ten pieces, diffusion was still restricted. Another approach was to use thinly sliced pituitaries, but they displayed poor tissue survival, presumably due to autolysis by the release of lysomal enzymes from ruptured cells (Farquar et al., 1975). These problems of diffusion restriction and cell death, along with the inability to make time course measurements of hormonal secretion

TABLE I

ANTERIOR PITUITARY AND HYPOPHYSIOTROPIC HORMONES

Anterior Pituitary Hormone	Hypophysiotropic Hormone
Thyrotropin	Thyrotropin-releasing Hormone
Adrenocorticotropin	Corticotropin-releasing Factor
Luteinizing Hormone	Gonadotropin-releasing Hormone
Follicle Stimulating Hormone	Gonadotropin-releasing Hormone
Growth Hormone	*Growth Hormone-releasing Factor
	Growth Hormone-release-inhibiting Hormone (somatostatin)
Prolactin	*Prolactin-releasing Factor
	*Prolactin-release-inhibiting Factor

*Existence presumed, structure not yet elucidated

and variability in responses of hemi-pituitaries of variable cell composition, limited the researcher (Nakano et al., 1976). The need for an improved in vitro technique became apparent. Such a technique was soon developed following the work of Rodbell (1964) who used enzymes to prepare suspensions of isolated fat cells to study the effects of insulin-mediated glucose transport across the cell membrane. Following this lead, Portanova et al. (1970) reported the successful enzymatic dispersion of anterior pituitary cells which retained their responsiveness to hypothalamic extracts. Many different agents were tried for cell dispersion (e.g., trypsin, hyaluronidase, neuraminidase, collagenase and EDTA) (Hopkins and Farquar, 1973; Moriarty et al., 1978; Vale et al., 1972) in order to find the dispersion method which would yield cells with secretory responses most like those found in vivo. The culture of dispersed cells has been used to investigate the secretion of the six anterior pituitary hormones, and the results obtained using this method have been accepted as valid (Pantic, 1975).

The cell dispersion technique provides a means of obtaining a large number of homogeneous cell populations in which to examine the effects of various agents or manipulations which would not be possible using randomly selected quartered or hemi-pituitaries of unacceptable cell heterogeneity (Farquar et al., 1975; Hopkins and Farquar, 1973; Vale et al., 1972).

Another benefit of cell dispersion is related to the different cell types found in the anterior pituitary. It is known that pituitary thyrotrophs, TSH secreting cells, are clustered in the center portion of the pituitary gland. When whole pituitary glands are cultured, the

thyrotrophs tend to be sequestered from direct contact with secreta-
gogues by the more exterior cells (Nakane, 1975). Cell dispersion
eliminates this restictive barrier of cells and allows the thyrotrophs
full exposure to the culture medium.

Cell culture methods have continued to be important for the study
of pituitary cells in vitro; however, the method was augmented by a
modification introduced in 1967 by Tait et al. This group of researchers
equipped an adrenal tissue culture with a continuous media flow to
observe the acid-stimulated release of aldosterone. Cell exposure to
secretagogues could be easily and accurately introduced into the medium
as a "pulse" and the response observed in a dynamic fashion. This
continuous flow system is presently known as the superfusion system.

The superfusion system was first used for the study of anterior
pituitary hormone secretion by Serra and Midgely (1970). Adding hypo-
thalamic extract to the medium superfusing whole pituitary glands
resulted in the secretion of luteinizing hormone, a dynamic secretory
response never before recorded. The superfusion method made possible
the collection of culture media throughout the course of cell stimula-
tion with various agents. Collecting small fractions of this superfusion
media permitted the determination of the hormone secretory profile that
included rates (grams/minute), response time, duration of response,
and amplitude of response.

The anterior pituitary cell superfusion system provided informa-
tion which approached that obtained with in vivo methods and avoided
some of the complexities of an intact, in vivo system, such as negative
feedback, circadian rhythms, and hypothalamic inhibitors. After the

cells have equilibrated with the continuous flow of fresh nutritive media, the administration of secretagogues (agents which stimulate hormone secretion) is permitted without mechanically disturbing the cells. The possible inhibition of hormone secretion attributed to negative feedback is greatly reduced as the secreted hormones and waste products are not permitted to remain in contact with the cells for any significant period. The dynamics of hormone release are easily measured by assaying the fractions of superfusate collected for hormone content.

Thyrotropin Releasing Hormone

The central nervous system had been long suspected of having a controlling influence on anterior pituitary function, but it was the report by Greer (1952) that provided more convincing evidence. He concluded that the hypothalamus mediated the secretion of thyroid stimulating hormone (TSH) by his demonstration that normal increases in plasma TSH levels following dietary supplements of propylthiouracil did not occur in rats which had had electrolytic lesions in the area of the hypothalamus. Experiments designed to study hypothalamic control of the pituitary-thyroid axis continued for the next ten years with interest primarily centered around the hypothalamic-hypophyseal system and the investigation of thyrotropin release (Greer, 1960).

The first evidence for a specific hypothalamic hypophysiotropic factor was obtained by Schally et al. (1966) using hypothalamic extracts to stimulate TSH secretion in vivo. Later, Wilber and Porter (1970) reported thyrotropin releasing activity in hypophyseal portal

blood. This evidence lead ultimately to the elucidation of the structure of porcine (Nair et al., 1970) and ovine (Burgus et al., 1970) thyrotropin releasing hormone (TRH). Thyrotropin releasing hormone is a tripeptide of pyroglutamyl-histidil-proline amide (m.w. 362) (Nair et al., 1970). This tripeptide has been synthesized and has been shown to possess all the biochemical activities of the natural peptide. As the first hypothalamic releasing factor to be isolated and characterized, the elucidation of TRH was a landmark in the field of neuroendocrinology.

The availability of synthetic TRH has led to numerous investigations of TRH activity. It has been well accepted that TRH stimulates the secretion of TSH in vivo. Many investigators have demonstrated TSH secretion in response to TRH stimulation in both in vivo and in vitro systems (Connors and Hedge, 1981; Dannies and Markell, 1980; Gershengorn et al., 1980; Martin et al., 1977; and Vale et al., 1967). The potency of TRH induced TSH secretion has been estimated to be 100,000 fold (Martin et al., 1977). Thyrotropin releasing hormone has been found in man, pig, rat, hamster, chicken, frog, snake, salmon, and sea lamprey. This evidence indicates that TRH occurs widely among the vertebrate classes (Jackson and Reichlin, 1974).

Control of Thyrotropin Secretion

Secretion of TSH from the anterior pituitary is generally accepted to be stimulated by TRH from the hypothalamus (Figure 1). Regulation of TRH stimulated TSH secretion is uncertain, but has been proposed to be accomplished by three possible mechanisms: inhibition of TSH
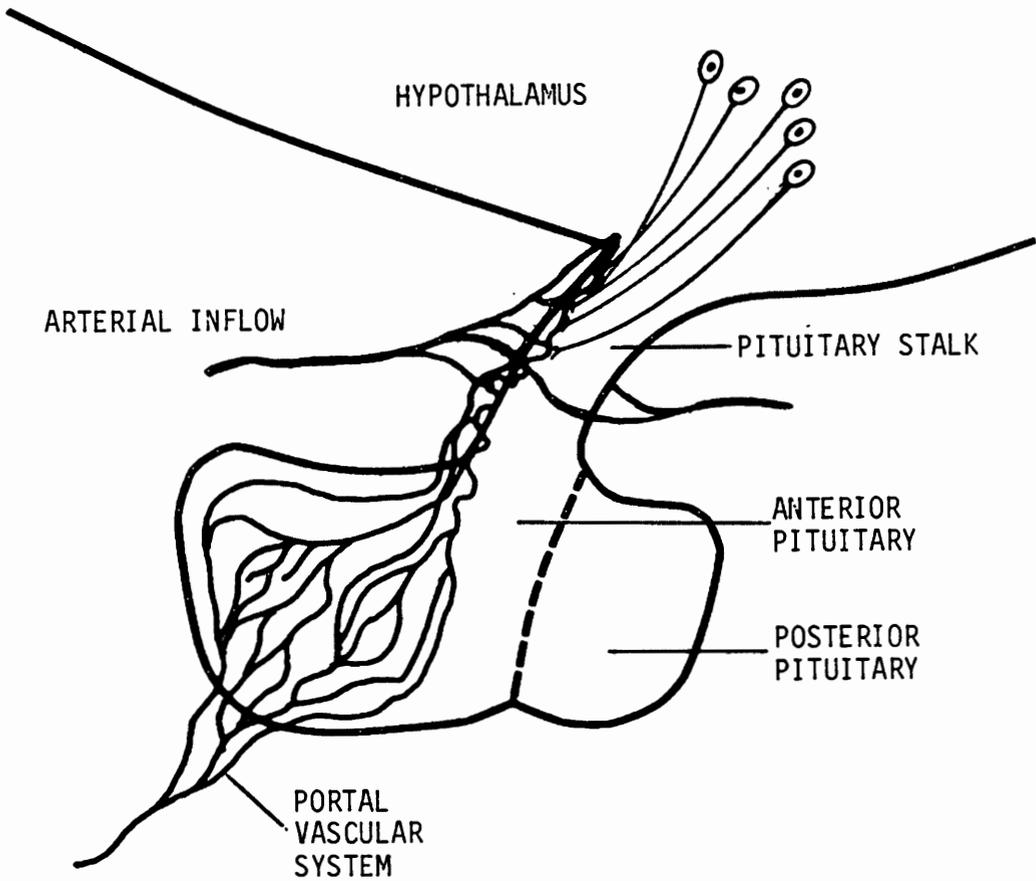
Figure 1.   The hypothalamic-hypophyseal portal system.

secretion by thyroid hormone feedback (Chopra et al., 1978), suppression by somatotropin release-inhibitory hormone (somatostatin) (Snow et al., 1978), or the down-regulation of specific TRH receptors on the thyrotroph cell surface (Peck and Gershengorn, 1980).

The pituitary-thyroid axis has served the science of endocrinology as a favorite example of a negative-feedback self-regulating system (Martin et al., 1977). The total regulatory scheme, as shown in Figure 2, involves the interaction of hormones from the hypothalamus, anterior pituitary, and the thyroid gland. First, TRH is released from the hypothalamus, is bound to receptors on the thyrotrophs, and elicits the release of TSH into the blood. Thyrotropin stimulates thyroid gland metabolism and secretion of the thyroid hormones, thyroxine (T4) and triiodothironine (T3). The thyroid hormones in the blood then "feedback" to the pituitary gland, and possibly the hypothalamus, to inhibit TSH secretion (Chopra et al., 1978). The negative feedback effect of T4 and T3 on TRH stimulation appears to be the primary control mechanism of TSH secretion (Martin et al., 1977).

Somatostatin is recognized as a specific inhibitor of growth hormone secretion but has also been shown to inhibit the secretion of both TSH and prolactin (Chopra et al., 1978). The effectiveness of somatostatin as an inhibitor of TSH secretion is limited in that while somatostatin will decrease the responsiveness of thyrotrophs to TRH stimulation, it will not decrease the basal secretion of TSH (Drouin et al., 1976). If somatostatin plays a role in the regulation of TSH secretion, it is probably secondary to the T4/T3 feedback regulation described above.
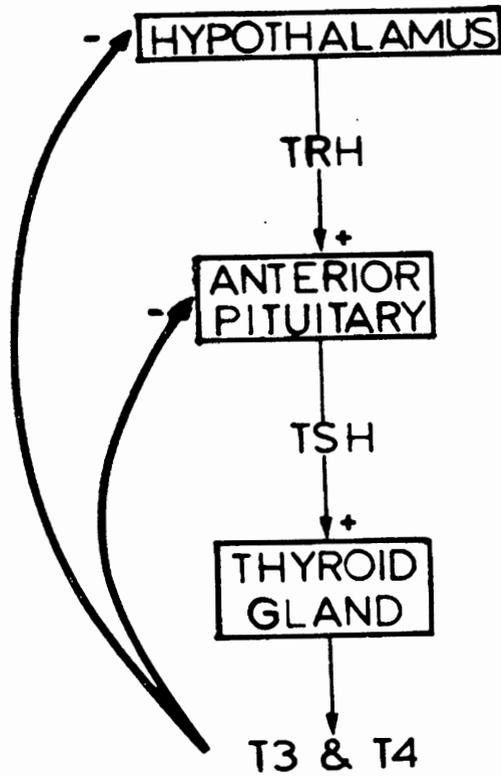
Figure 2. Diagram of the hypothalamic-pituitary-thyroid axis.
(+) = positive stimulation, (-) = negative stimulation.

A third mechanism of control proposed is associated with the number of TRH receptors on the thyrotroph. Data have been interpreted to give evidence that TRH regulates the number of its own receptors (Gershengorn, 1978; Hinkle and Tashjian, 1975). Knowing that one TRH receptor activated by one molecule of TRH has the capacity to affect the release of TSH 100,000 fold, it is easy to see that small decreases in the available TRH receptors would result in a very large decrease in TSH secretion.

Thyrotropin Releasing Hormone and the Second Messenger Theory

Thyrotropin releasing hormone stimulates TSH release by binding to an extracellular TRH receptor on the thyrotroph (Grant et al., 1973). Cyclic 3', 5' adenosine monophosphate (cyclic AMP) is believed to serve as an intracellular messenger of anterior pituitary hormone secretion (Labrie et al., 1978), and has been implicated as a second messenger of TRH-mediated TSH secretion (Moriarty et al., 1978; Steiner et al., 1970; Wilber et al., 1968). However, this role for cyclic AMP in TSH secretion is not shared by all investigators (Bowers, 1971; Gershengorn et al., 1980; Sundberg et al., 1976).

The second messenger theory for hormone release was first suggested after the discovery of cyclic AMP and the adenyl cyclase enzyme (Sutherland et al., 1968). According to this theory, the first messenger is an extracellular hormone which binds to a cell membrane receptor. The binding of the first messenger activates the intracellular, membrane-bound adenyl cyclase enzyme to convert adenosine triphosphate to cyclic AMP and inorganic phosphate. The newly formed

cyclic AMP is released from the membrane bound enzyme into the cyto-
plasm as a second messenger, carrying the "command" of the first
messenger. The cyclic AMP activates other enzymes in the cell (pro-
tein kinases). These protein kinases, activated after binding to
cyclic AMP, mediate other specific cellular activities (Figure 3).
The cyclic AMP is quickly converted to the inactive 5'-AMP by the
enzyme phosphodiesterase, and the message of the second messenger is
ended (Steer, 1975).
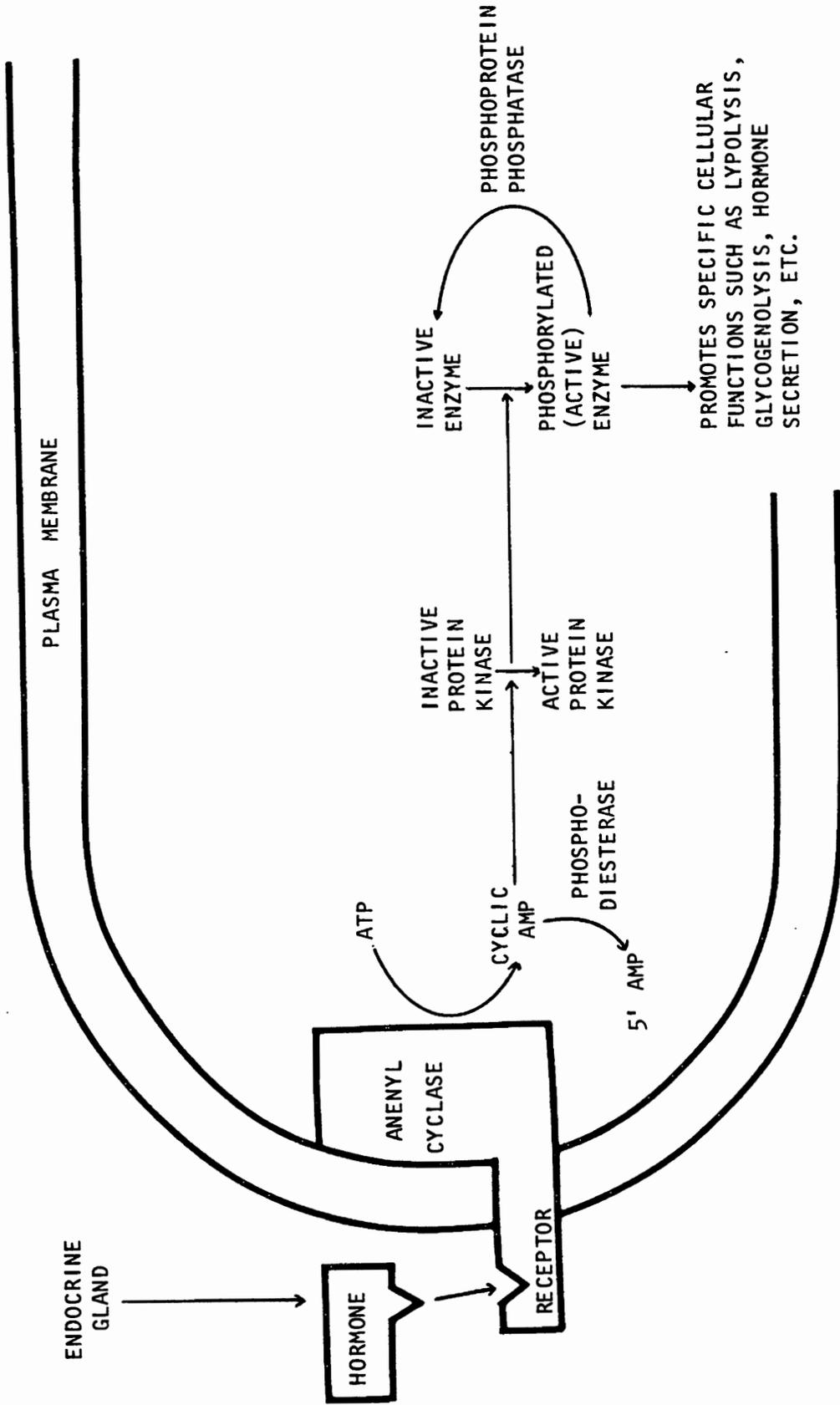
Figure 3.   The Adenyl cyclase system.

CHAPTER III

MATERIALS AND METHODS

Animals

Pituitary cells used for the superfusion experiments were
obtained from Lewis male rats (Charles River, 60-130 days old), kindly
provided by Dr. Wesley Bullock. The rats were housed in the Oregon
Health Sciences University School of Dentistry Animal Care and Re-
search Facility which was maintained at a constant temperature of $12^{\circ}C$,
with a 12 hr light/dark cycle; the rats were fed rat chow (Wayne
Lab Blox F-6) and tap water ad libitum.

Tissue Collection and Cell Preparation

Media used for the superfusion experiments were Hankes-HEPES
buffer (H-H) and Krebs-Ringer buffer (KRB) with glucose and bovine
serum albumin (BSA) (See Appendix for chemical composition). Both
buffers were maintained at a constant temperature of $25^{\circ}C$ and pH 7.3,
and equilibrated with a 95% O_2 - 5% CO_2 atmosphere.

The rats were killed by sudden decapitation within twenty seconds
after removal from their cages. The pituitaries were quickly removed
and washed three times in H-H buffer. Posterior lobes were removed
and the anterior lobes washed a fourth time. The anterior lobes were
minced into approximately twenty pieces each with scissors in a sili-
conized watchglass containing H-H buffer. The minced pieces were then

suspended in 5.0 ml H-H buffer containing collagenase (3.0 mg/ml, Sigma Type II-S) and BSA (5.0 mg/ml, Sigma A-8022). This pituitary cell suspension was then agitated (60 cycles/min in a Dubnoff Metabolic Shaking Incubator, GCA Corp.) for 40 minutes. The cell suspension was mechanically agitated every 10 minutes by repeatedly drawing and expelling the suspension using a siliconized pasteur pipet. After 40 minutes of dispersion, the suspension was mixed with the pipet 100 times. The cell suspension was then filtered of connective tissue and debris using a fine nylon mesh (Nitex #102). The nylon mesh was then washed with 5.0 ml H-H buffer. After centrifuging the filtered cell suspension, the supernatant was aspirated and the pellet resuspended in 2.5 ml KRB.

To count the total number of cells, 50 ul of the cell suspension were added to 100 ul KRB and 100 ul diluting fluid. After mixing well, 25 ul of the suspension were deposited into a hemacytometer chamber. The four corner squares and the center square of the counting grid were counted. The following formula was used:

$$\frac{\text{total cells counted}}{2.0 \times 10^{-5} \text{ ml}} \times 5 = \text{no. cells/ml.}$$

Care was taken to count only nucleated pituicytes.

To assess the viability of the dispersed pituitary cells, the trypan blue dye exclusion procedure was followed as outlined by Merchant et al. (1964). A 0.5 ml volume of cell suspension (diluted to $10^5 - 2 \times 10^5$ cells/ml) was placed in a 12 x 75 mm glass test tube with 0.1 ml of 0.4% trypan blue (GIBCO). The cell suspension was mixed thoroughly and allowed to stand for five minutes. The number of viable

cells was determined as outlined by Davidson and Henery (1969) using 25 ul of the trypan blue/cell suspension. The test is based on the ability of the viable cells to exclude the dye; therefore, the formula for calculating the viability is as follows:

$$\frac{\text{No. clear cells}}{\text{total no. cells}} \times 100 = \% \text{ viability.}$$

Superfusion Method

The superfusion column was constructed using a 3 cc disposable syringe (Monoject) for a column (Figure 4). The plunger in the column was replaced with an inverted 1 cc syringe trimmed to 2 cm. The rubber gasket from the unused plunger of the 3 cc syringe was removed, a 6 mm hole cut through its center, and cemented to the 1 cc barrel to insure a tight seal. Eighteen gauge needles were put in place on the leur tips at each end of the column, which were in turn attached to tygon tubing. Parafilm was used to seal all joints on the column to prevent leakage.

The cells rested on an inert bed of Sephadex G-15 (Pharmacia Fine Chemicals) which had been allowed to swell 72 hours in phospho-saline buffer before use. A small circle (1 cm diameter) of grade 934 AH glass fiber filter paper (Reeve Angel) placed at the bottom of the column provided a base for the Sephadex to assure stability of the bed while not impeding the flow of superfusion medium. The Sephadex was poured into the column to a height of 1.5 cm and washed with approximately 10 ml KRB. After washing and allowing the KRB to drain to the top of the Sephadex bed, 10^7 viable cells were carefully applied.
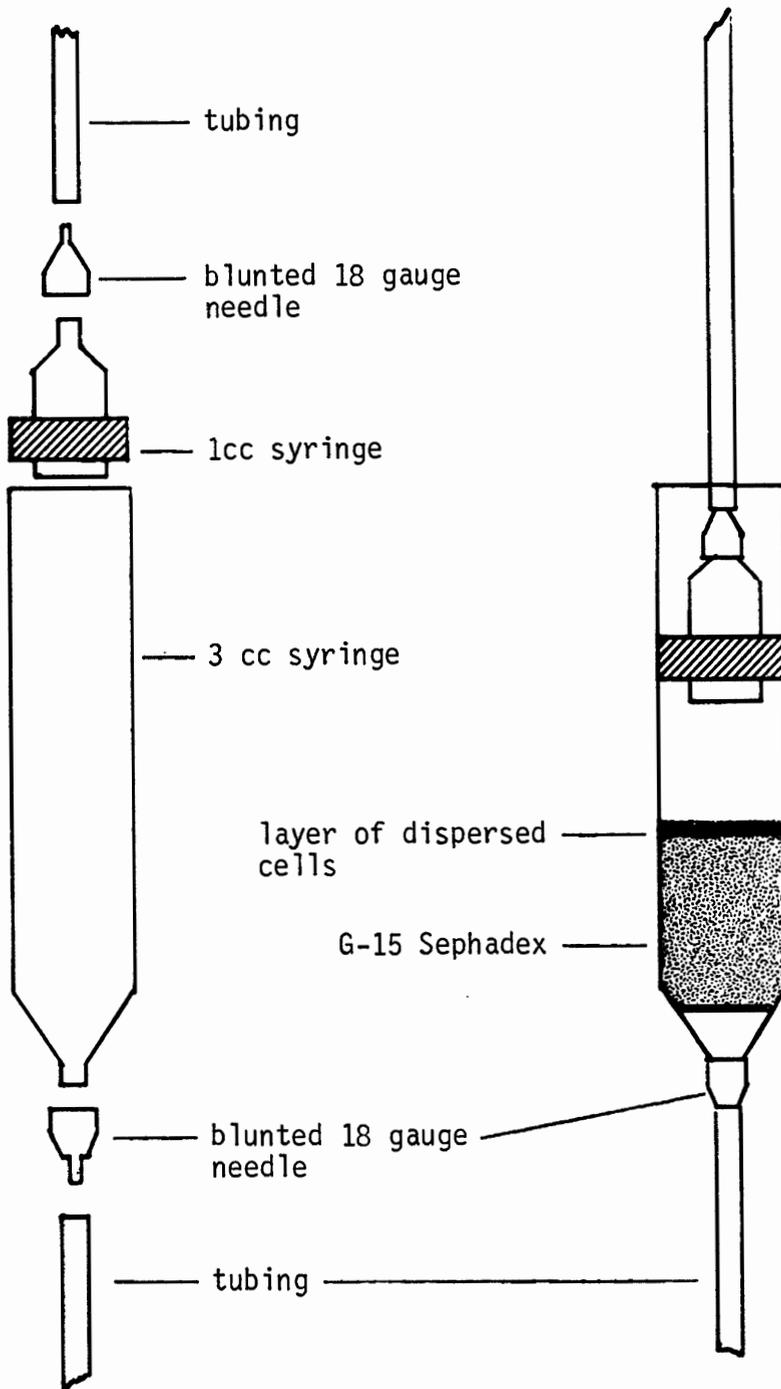
Figure 4. The superfusion column.

Once the cells settled, forming a layer on the Sephadex, the 1 cc syringe was carefully inserted, the column sealed and checked for leaks, and superfusion begun.

The flow rate of the KRB superfusion medium was maintained at 0.5 ml per minute, which is comparable to the flow rate of the hypophyseal portal blood flow found in vivo (Goldman and Sapirstein, 1958). This rate of flow was established using a Sage Tubing Pump (Model 375-A) and was verified at the beginning and ending of the experiment. Superfusion medium was collected in 1.0 ml fractions (2 minute intervals) using a Gilson fraction collector (Figure 5). Fractions were frozen and stored at -20°C for hormone analysis at a later time.

Radioimmunoassay of Thyrotropin

The radioimmunoassay (RIA) procedure followed was a double antibody procedure outlined by the NIAMDD with some minor alterations (See protocol in Appendix). Thyrotropin for iodination (TSH-I-4), standards (TSH-RP-1), and TSH antibody were a generous gift of the NIAMDD Rat Pituitary Agency. Sheep anti-rabbit serum for the second antibody was purchased from Gene Stone, Medford, Oregon.

Quality control for the RIA procedure followed the method described by Rodbard (1974). Rat plasma pool samples were assayed in triplicate for each RIA. The parameters monitored were the within-assay and between-assay variation. Within-assay and between-assay variabilities were 13.9% and 22% respectively, both considered acceptable for RIA of TSH (Fukuda et al., 1975; Russel et al., 1975).
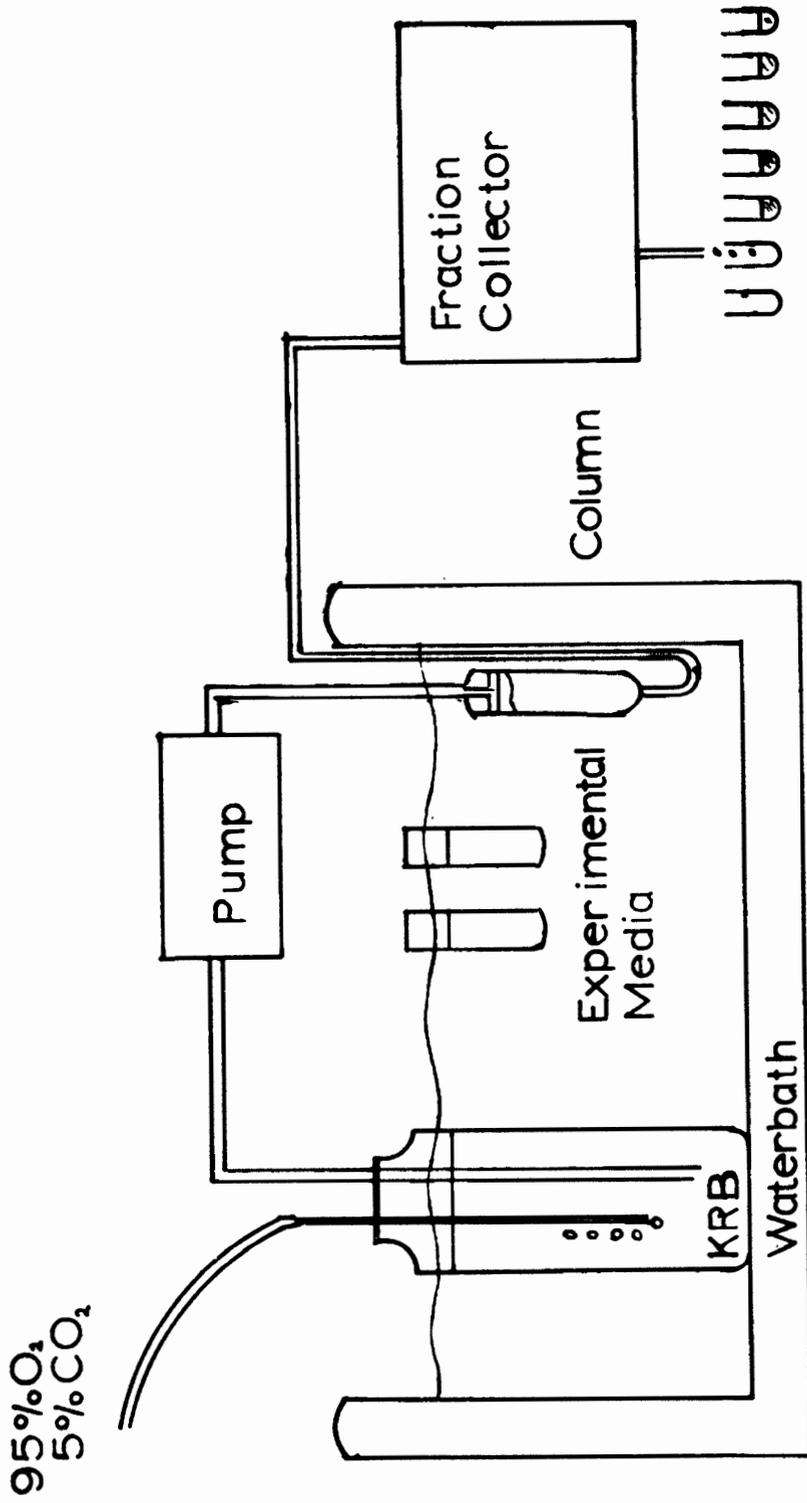
Figure 5. Schematic illustration of the superfusion apparatus.

Intracellular TSH Content

The intracellular concentration of TSH was determined by using 50 ul of the cell suspension, designated the pre-superfusion sample, diluted with 0.95 ml distilled water, and frozen for later assay. Post-superfusion TSH cell content was determined after removing the entire contents of the superfusion chamber (Sephadex and cells) with 2.0 ml distilled water, and frozen.

Thyrotropin Flow Through the Superfusion Column

The specific design and construction of the superfusion system used to study TSH secretion was new; thus, it was first necessary to determine if TSH flows freely through the superfusion system and is not slowed or trapped by the Sephadex or pituitary cells. This was tested by superfusing TSH (0.5 ug/ml) through two Sephadex columns, one containing anterior pituitary cells, the other containing Sephadex only. The results of these experiments are shown in Figure 6 and Table II. Elevated levels of TSH appear in fractions collected immediately following superfusion of TSH and return to baseline levels within four minutes after cessation of TSH superfusion in both columns. The flow of TSH is neither retarded, nor is TSH retained by the superfusion system. An "ideal elution curve" is added to Figure 6A to indicate the expected TSH content of the fractions collected during TSH superfusion, assuming no restriction of TSH flow and no within-assay variation in the TSH-RIA.

Verification of Stable, Basal Thyrotropin Secretion

Before a response to a secretagogue can be measured in the superfusion system, it is essential to first establish a constant basal level
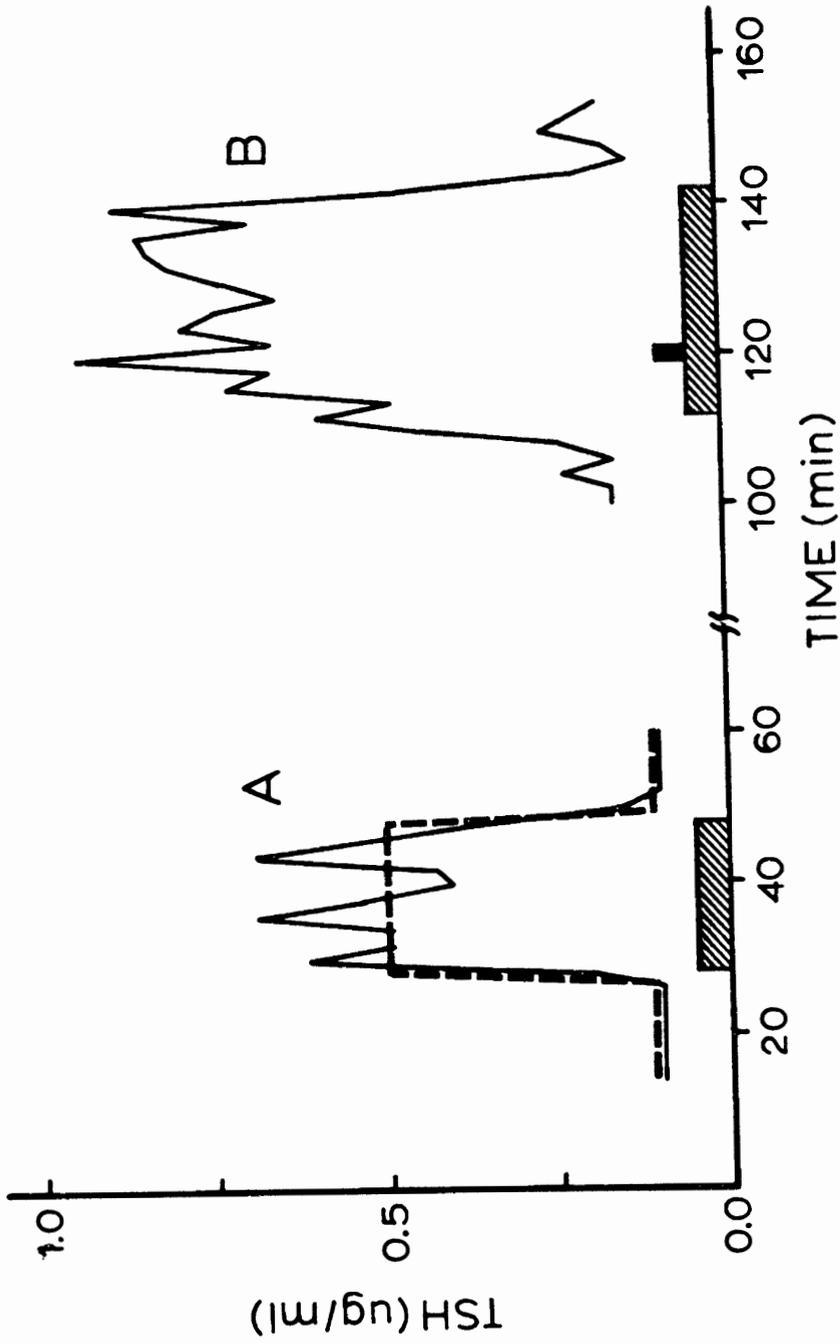
Figure 6. TSH content of superfusate fractions collected every two minutes. TSH (0.5 ug/ml) was superfused through a column containing G-15 Sephadex only (A) and one containing Sephadex with 10.4 million anterior pituitary cells (B). The dotted line in Figure A is the "ideal elution curve" of superfused TSH.

TABLE II

TSH SUPERFUSION AND RECOVERY THROUGH SEPHADEX COLUMNS
WITH AND WITHOUT ANTERIOR PITUITARY CELLS

Column	ug TSH Superfused	ug TSH Recovered	Variation*
Sephadex alone	2.00	1.85	7.5%
	2.51	2.80	11.5%
Sephadex with cells	1.96	1.73	11.5%
	2.45	2.67	8.9%

*Within-assay variation for the TSH RIA was 13.9%.

of TSH secretion. The TSH response to secretagogues can then be assessed by comparison with the baseline. For this reason, the secretion of TSH from non-stimulated cells was determined, as shown in Figure 7. Basal TSH secretion from superfused anterior pituitary cells was determined from the time the cells were layered on the column and the flow of medium begun, until 140 minutes. Thyrotropin secretion during the first 10 minutes was 3.9 ug TSH/ml and established a relatively stable basal level (0.125 - 0.25 ug TSH/ml) after 90 minutes of superfusion. Based on these data and the published data of others, all superfusion experiments were routinely given a pre-incubation period of 180 minutes to establish secretory equilibration. Fractions were collected during the last 20 minutes of the pre-incubation period of each experiment in order to confirm baseline stability.

25
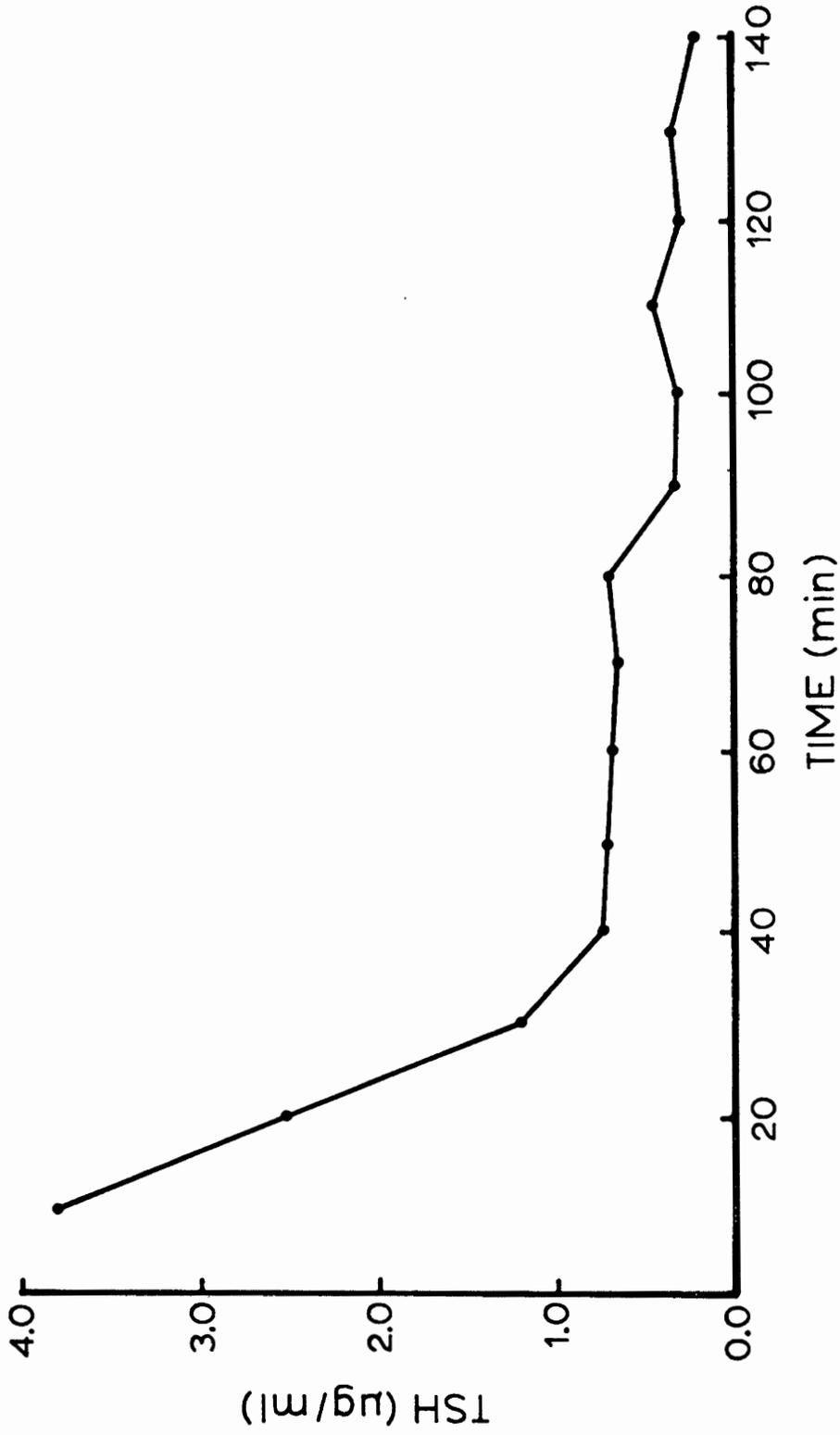
Figure 7. Non-specific TSH secretion from superfused, dispersed anterior pituitary cells. TSH secretion during ten-minute intervals from the time the cells were applied to the column is shown.

CHAPTER IV

RESULTS

Thyrotropin Secretion in Response to Thyrotropin Releasing Hormone

As the accepted physiologic stimulator of TSH secretion, TRH-mediated TSH secretion was examined using the superfusion system. Thyrotropin responsiveness to a two-minute pulse of TRH (10^{-8} M) in the superfusion system is immediate, as indicated in Figure 8. Thyrotropin secretion rose to 0.36 ug/ml above baseline and returned to baseline levels within four minutes of cessation of TRH superfusion.

Repeated Stimulation of Anterior Pituitary Cells with Thyrotropin Releasing Hormone

The effect of repeated TRH stimulation and TSH responsiveness was examined using the superfusion system. Two columns were prepared to be superfused in parallel. The same pools of KRB and TRH (10^{-8} M) were used for each. The two columns (A and B) each contained 10^7 anterior pituitary cells from the same cell pool. Each cell preparation received two-minute pulses of TRH repeatedly during the course of the experiment. Results are shown in Figures 9 and 10. The cells in column A (Figure 9) were stimulated with TRH at 0, 1, 2, and 3 hours. After the initial TRH pulse, total ug TSH secreted in response to TRH at 1, 2, and 3 hours decreased 26%, 20%, and 17.3% respectively. The maximum amplitude of the TSH response peak dropped 39.8% after the first TRH pulse but remained stable thereafter (Table III). Column B
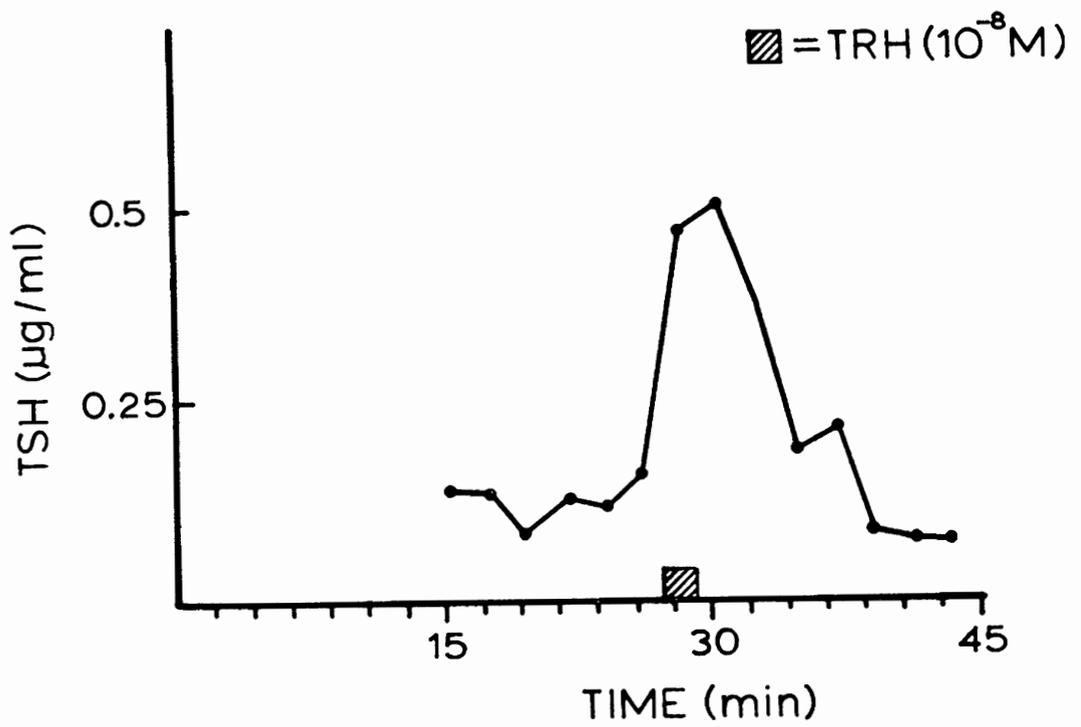
Figure 8. TSH secretion in response to 10^{-8} M TRH superfused for two minutes.
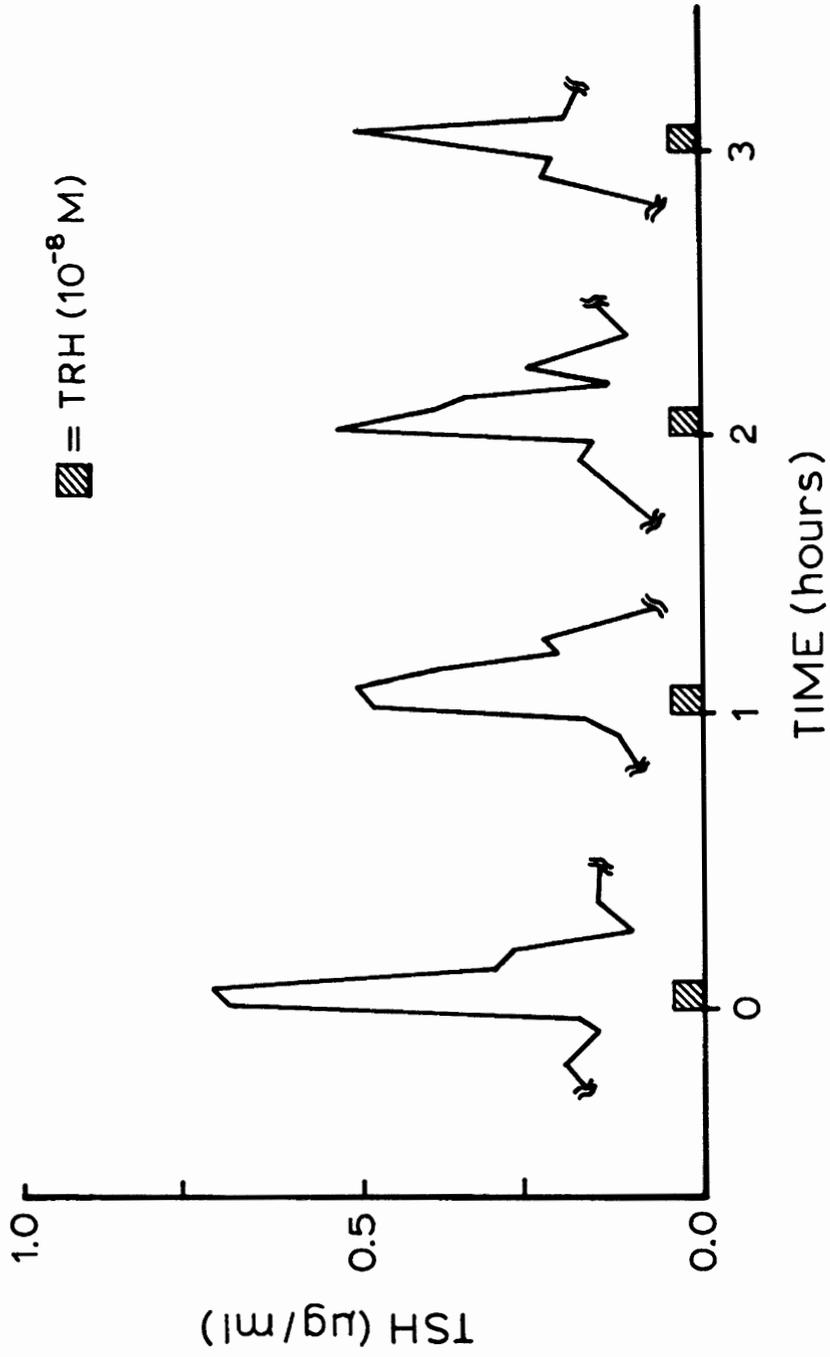
Figure 9. TSH secretion in response to two-minute pulses of 10^{-8} M TRH. TRH was superfused at 0, 1, 2, and 3 hours.
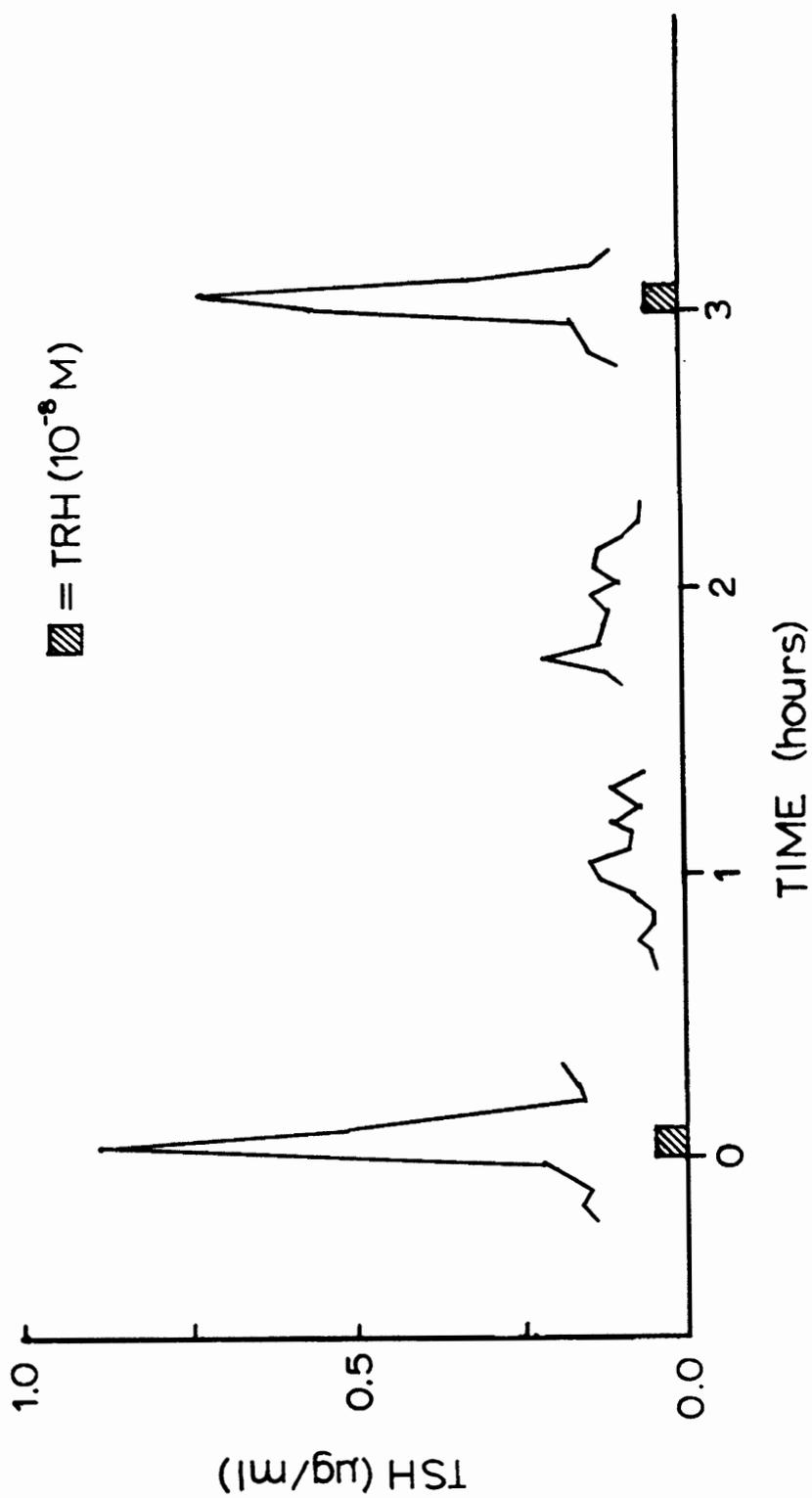
Figure 10. TSH secretion in response to two-minute pulses of 10^{-8} M TRH. TRH was superfused at 0 and 3 hours. Basal TSH secretion at 1 and 2 hours has been included.

TABLE III

PEAK AMPLITUDE AND TOTAL TSH RESPONSE TO TWO-MINUTE PULSES
OF 10^{-8} M TRH FROM SUPERFUSED ANTERIOR
PITUITARY CELLS

Column	Time of TRH pulse (hr)	Peak TSH response (ug/ml)	Total TSH response (ug)
A	0	0.59	1.47
A	1	0.36	1.09
A	2	0.38	0.87
A	3	0.35	0.71
B	0	0.70	1.12
B	3	0.59	1.07

(Figure 10) was pulsed with TRH at 0 and 3 hours. Although total ug TSH secreted after the first TRH pulse in column B did not change greatly, the maximum amplitude of the TSH peak response declined 15.7%.

The TSH peak response profiles from column A and column B are shown in Figure 11. The responses are superimposed to facilitate comparing the height and breadth of each peak and to more easily visualize the area under the peak curve which represents the total TSH secreted. While the amplitude of the peak responses remained stable for column A at 1, 2, and 3 hours, the duration of the response decreased with each successive TRH pulse. On the other hand, column B showed no change in response duration while the peak amplitude decreased.

Shortloop Feedback Regulation of TSH Secretion

Shortloop feedback regulation of TSH secretion was investigated using the superfusion system. Two questions concerning the shortloop feedback phenomenon were addressed. First, would TSH shortloop feedback inhibition of TRH-mediated TSH release be dependent on the time in which the TSH and TRH are presented to the cells? And second, is there a TSH dose which is critical to the shortloop feedback effect?

To determine if a time dependent relationship exists between TSH and TRH presentation which would result in a shortloop feedback inhibition of TSH secretion, the following procedure was followed. Anterior pituitary cells were superfused with two-minute pulses of TRH (10^{-8} M) in the presence of TSH (0.5 ug/ml) (Figure 12). Thyrotropin releasing hormone was presented to the cells during the final two minutes of a ten-minute TSH superfusion (Chart A of Figure 12) and immediately following a ten-minute TSH superfusion (Chart B of Figure
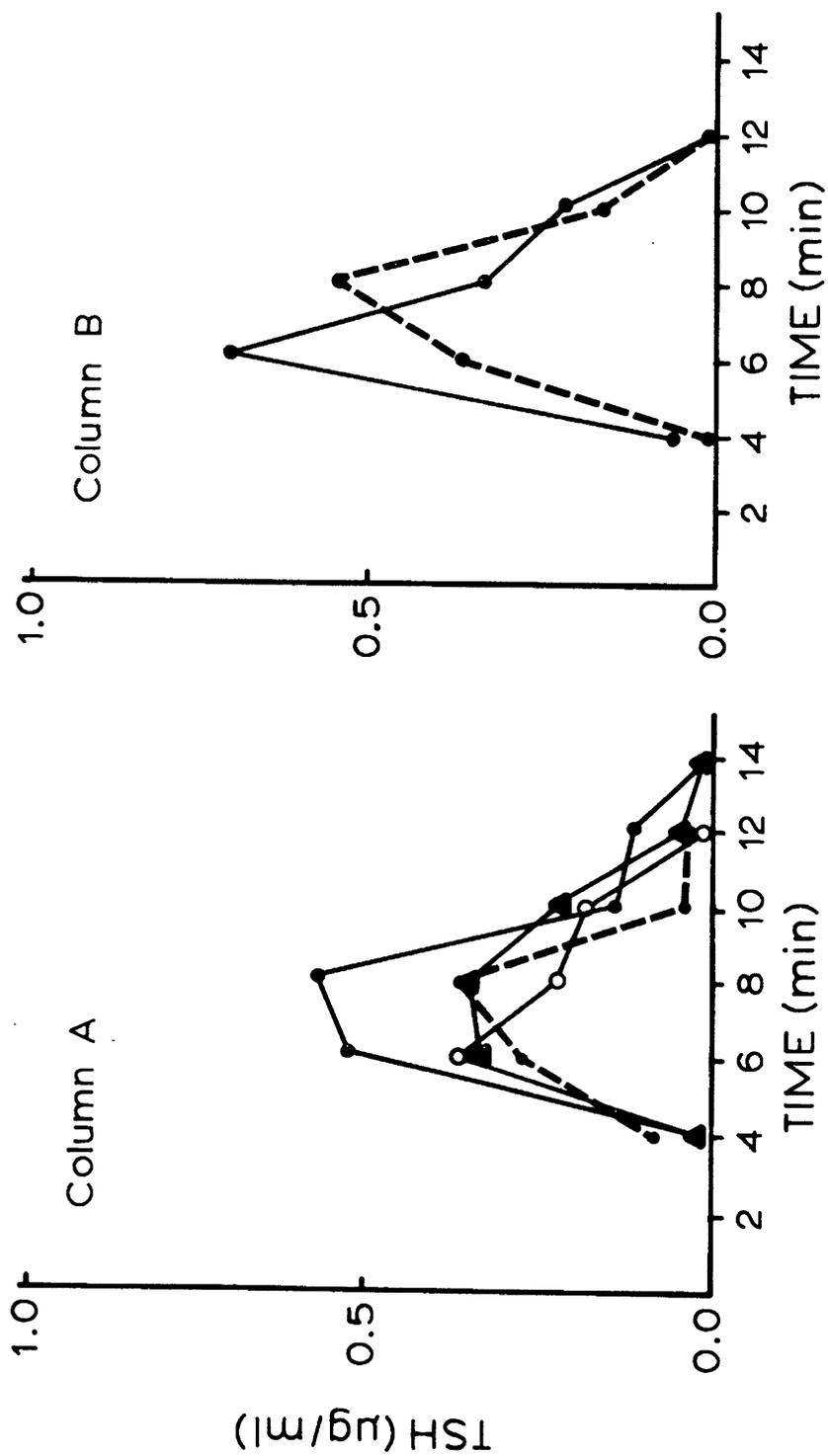
Figure 11. Superimposed profiles of TSH peak responses to two-minute 10^{-8} TRH pulses. These peak profiles are from Figures 9 (Column A) and 10 (Column B). The four Column A peaks represent the TSH secretion from TRH pulses at 0 (•——•), 1 (▲——▲), 2 (o——o), and 3 (•–––•) hours. The two peaks from Column B represent the same TRH pulses administered at 0 (•——•) and 3 (•–––•) hours.
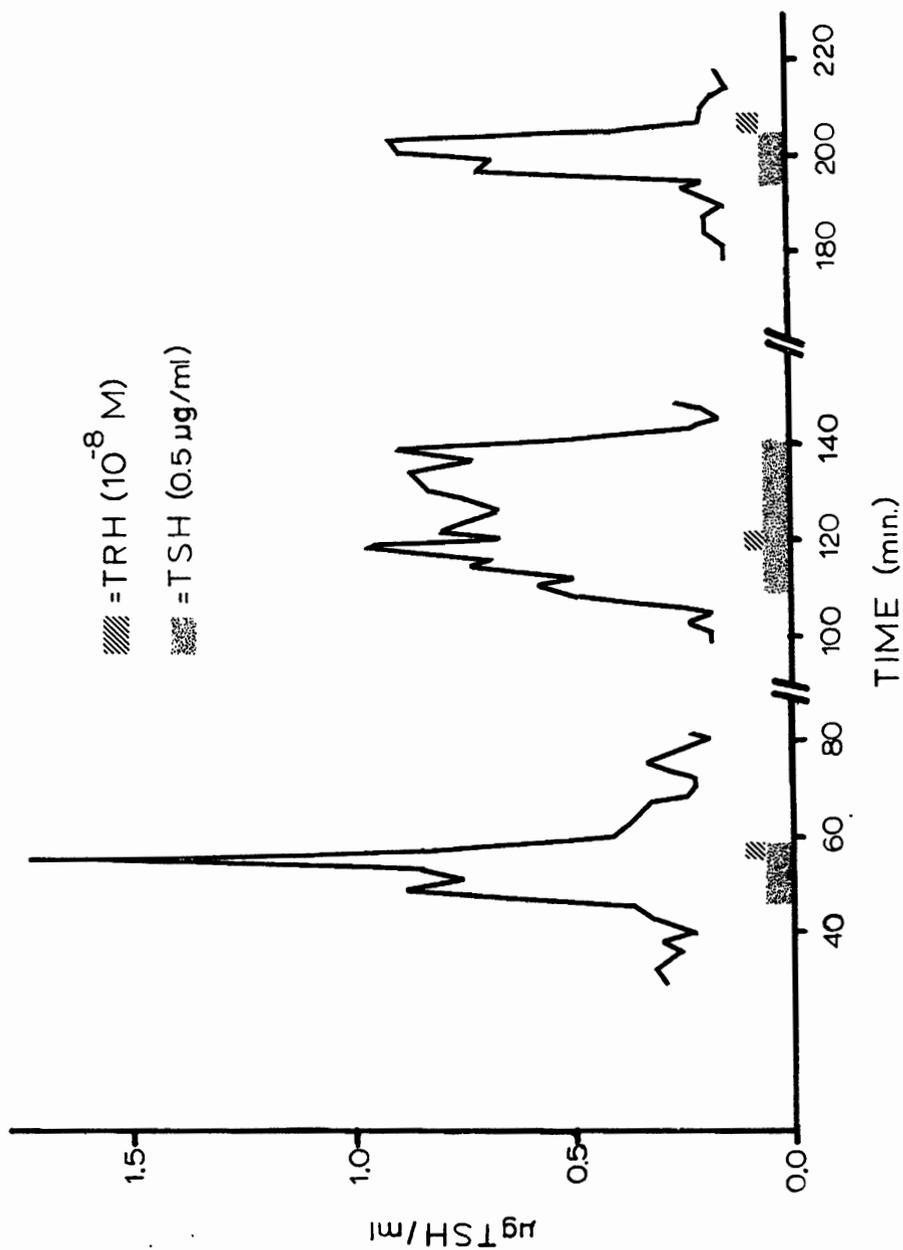
Figure 12. TSH responses to 10^{-8} M TRH in the presence of 0.5 ug TSH/ml. Two minute pulses of TRH were superfused during the final two minutes of TSH superfusion (12A), after ten minutes of a thirty-minute TSH superfusion (12B), and immediately following TSH superfusion.

12). In both cases, the cells are responsive to TRH. However, when a two-minute pulse of TRH was presented to the cells at ten minutes after a TSH superfusion for thirty minutes, the TSH response was abolished (Chart B of Figure 12). These data indicate that TRH-mediated TSH release can be blocked by maintained high levels of TSH above baseline secretion.

To determine if the inhibitory effect of TSH on the TRH-stimulated TSH secretion described above was dose-dependent, three different concentrations of TSH (0.25, 0.5, and 1.0 ug/ml) were tested. Thyrotropin releasing hormone and TSH were administered following the protocol used in Chart B of Figure 12 above. Thyrotropin responsiveness to TRH was unchanged in the presence of 0.25 ug TSH/ml (Chart C of Figure 13). When TRH was administered in the presence of 0.5 ug TSH/ml, the TSH response to TRH was attenuated (Chart A of Figure 13). Finally, TSH at a concentration of 1.0 ug/ml totally abolished the TRH-stimulated TSH response (Chart B of Figure 13).

Superfusion of Dibutyryl Cyclic 3',5'-Adenosine Monophosphate

Cyclic adenosine monophosphate (cyclic AMP) has been reported to be a second messenger involved in the TRH-mediated TSH release from anterior pituitary cells. To examine the effect cyclic AMP may have on TSH secretion from superfused anterior pituitary cells, dibutyryl cyclic AMP (10^{-8} M) was superfused for two and ten minutes. In four experiments, no change in baseline TSH secretion was observed during dibutyryl cyclic AMP superfusion (data not shown).

Thyrotropin responses to:

☐ TRH (10^{-8} M)

■ TRH with 1.0 ug TSH/ml

▨ TRH with 0.5 ug TSH/ml

▩ TRH with 0.25 ug TSH/ml
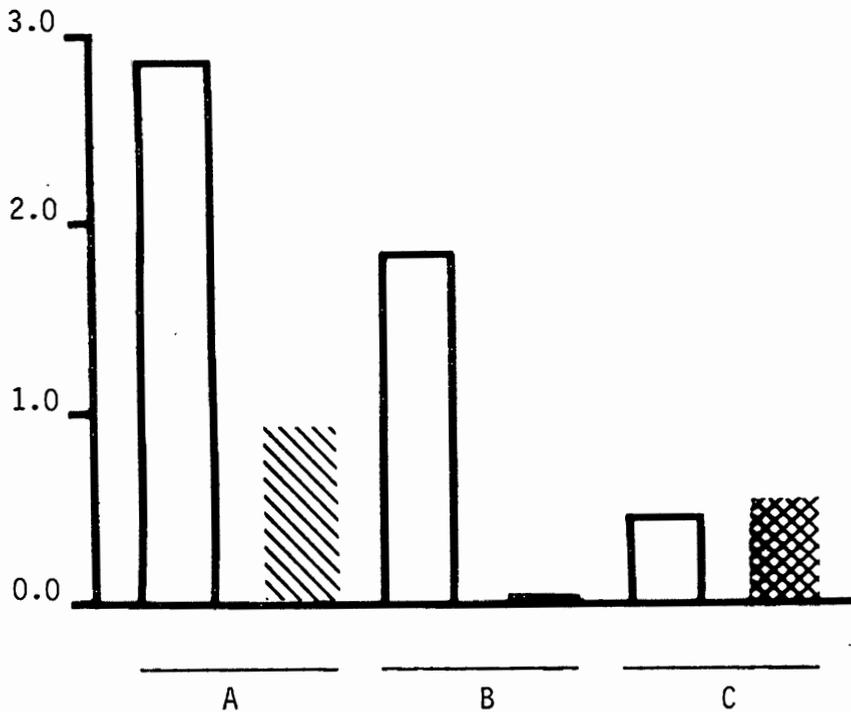
Figure 13. Thyrotropin secretion in response to TRH presented to cells alone or accompanied by different doses of TSH. When 0.5 ug TSH/ml was superfused with a 10^{-8} M TRH stimulus, TRH-mediated TSH secretion dropped 67% compared to TSH response to TRH alone (panel A). When 1.0 ug TSH/ml accompanied a TRH stimulus, TRH-mediated TSH secretion was totally blocked (panel B). When a TRH stimulus was presented in conjunction with 0.25 ug TSH/ml, no inhibition of TRH-mediated TSH secretion was observed (panel C).

Somatostatin as an Inhibitor of Thyrotropin Secretion

Somatostatin inhibition of TRH-mediated TSH release was examined next. Cells were stimulated with either TRH (10^{-8} M) or pituitary stalk-median eminence extract (SME, 1.0 or 0.5 U/ml) in the presence or absence of somatostatin (10^{-8} M). Pituitary cells were responsive when TRH alone was administered. However, if TRH were presented to the cells during somatostatin superfusion, no TSH response was observed (Figure 14). Anterior pituitary cells are responsive to both doses of SME when presented alone or in the presence of somatostatin (Table IV). These data indicate that somatostatin (10^{-8} M) will block TRH-mediated TSH release but has no effect on the SME-mediated TSH release.

Thyrotropin Secretion in Response to High Potassium Ion Concentration

Potassium, another secretagogue, was examined next in the superfusion system for its ability to stimulate TSH release from anterior pituitary cells. Dispersed cells superfused with potassium ion at twenty-five times the normal concentration, for two minutes, responded with an increase in total TSH secretion (1.34 ug) (Figure 15). The cells were then superfused with potassium in the presence of 10^{-8} M somatostatin. The TSH response to potassium in the presence of somatostatin was attenuated but not blocked (0.98 ug). When a third two-minute potassium pulse was presented, the total TSH secretion dropped to 0.37 ug. It should be noted that the baseline TSH secretion was also diminished after the somatostatin superfusion to levels below the detectable limit of the TSH-RIA.
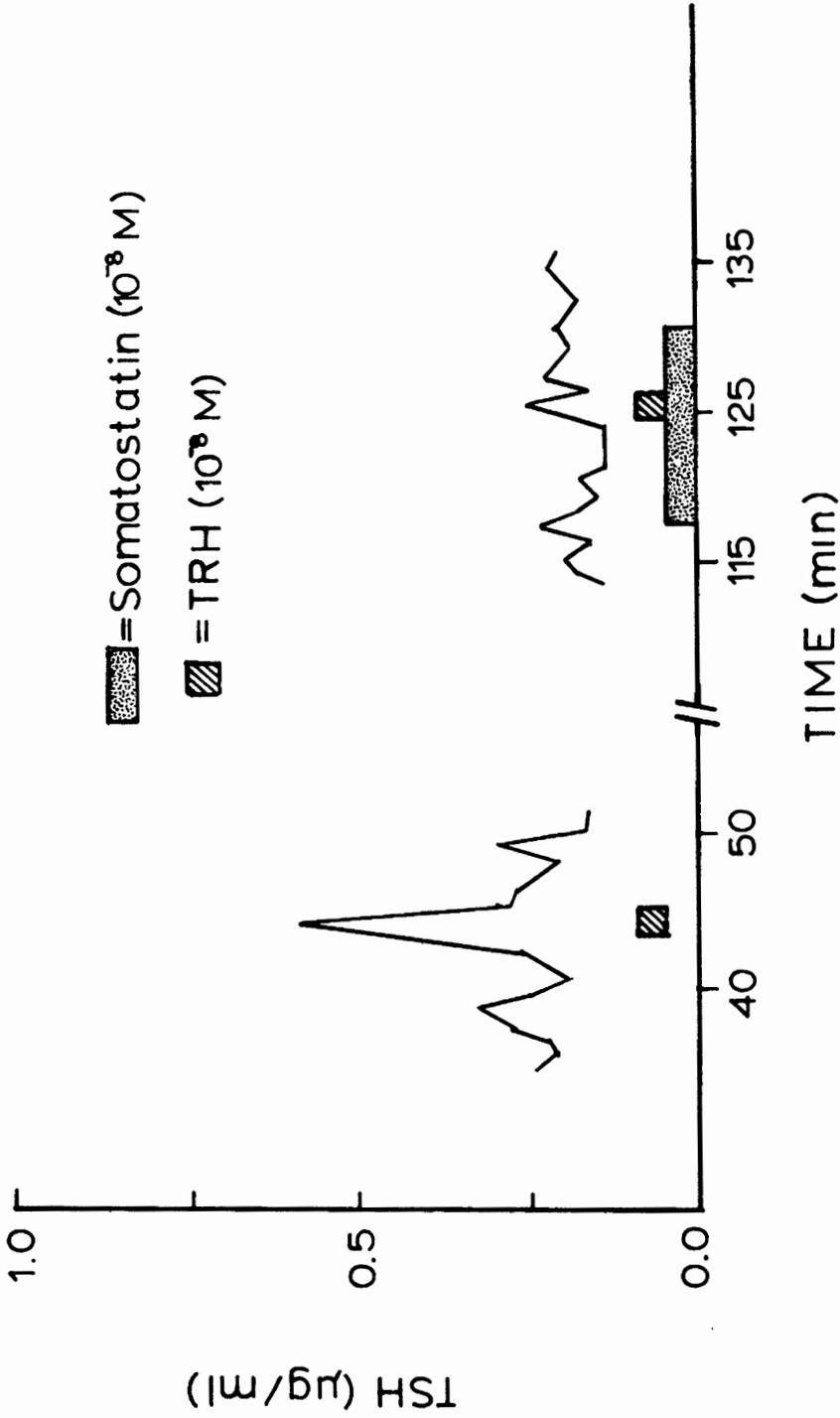
Figure 14. TSH secretion in response to 10^{-8} M TRH alone and in the presence of 10^{-8} M somatostatin.

TABLE IV

TOTAL TSH SECRETION IN RESPONSE TO PITUITARY STALK MEDIAN-EMINENCE
EXTRACT (SME) ALONE AND IN THE PRESENCE
OF 10^{-8} M SOMATOSTATIN

SME (U/ml)	Somatostatin (concentration)	Total TSH secreted* (ug)
1.0**	0	1.85
1.0	10^{-8} M	1.77
0.5	0	0.925
0.5	10^{-8} M	0.910

*One unit SME equals the extract obtained from one rat pituitary
stalk median-eminence.

**TSH responses are given as the total ug TSH secreted above
baseline in response to a two-minute SME pulse.
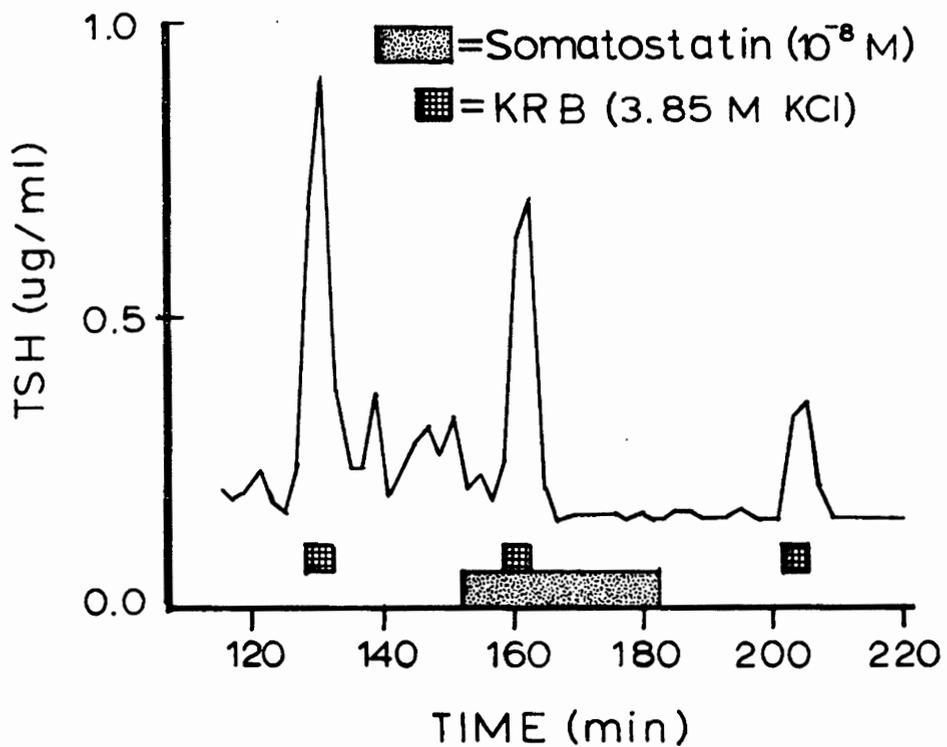
Figure 15. TSH secretion in response to 3.85 M KCl. Two-minute pulses of KRB which contained twenty-five times the normal concentration of KCL were given alone and in the presence of 10^{-8} M somatostatin.

Intracellular Thyrotropin Content

The intracellular TSH content of dispersed anterior pituitary cells was assessed by RIA to determine if the intracellular TSH reserves were exhausted. The results of two such assays are given in Table V. The pre-superfusion TSH cell content for the two super-fusions was 13.7 and 9.98 pg/cell. The cellular TSH content after superfusion dropped to 1.99 and 1.22 pg/cell. This reflects an 85.47% and an 87.77 drop in intracellular TSH.

TABLE V

THE INTRACELLULAR TSH CONTENT OF DISPERSED ANTERIOR PITUITARY
CELLS BEFORE AND AFTER SUPERFUSION

Pre-superfusion TSH content (pg)	Post-superfusion TSH content (pg)	Superfusion Period* (hrs)	Percent Decrease
13.70	1.99	8.5	85.47
9.98	1.22	6.8	87.77

*Cells superfused 8.5 hours received six, two-minute pulses
of 10^{-8} M TRH, while the cells superfused 6.8 hours received
six, two-minute pulses of 3.85 M KCl and twenty-minutes of
10^{-8} M somatostatin.

CHAPTER V

DISCUSSION

The superfusion system is an in vitro system which simulated,
to an extent, the continuous flow situation found in vivo. This
method has been utilized previously for the study of anterior pitui-
tary hormones, including thyrotropin (Mulder and Smelic, 1977; Swano
and Kokubu, 1977; Dowd, et al., 1975; Connors, et al., 1981). The
TSH secretory response to TRH (10^{-8} M) in this current study are com-
parable to those previously measured in a superfusion system (Connors,
et al., 1981). Therefore, it has been demonstrated that the pituitary
cell superfusion system used for this study is responsive to TRH and
is physiologically comparable to those of other laboratories.

The magnitude of TRH-mediated secretion from superfused, dispersed
anterior pituitary cells decreased with repeated TRH stimulation. In
all superfusion experiments conducted for this study, the amplitude of
the initial TSH peak was greater than all subsequent TSH responses. And
while the amplitude of the later TSH responses remains fairly constant,
the total micrograms TSH secreted per TRH pulse declines (Table III).
These observations are in agreement with those of Nakano, et al. (1976)
and Vale, et al. (1972) who reported a decrease in TSH responsiveness
to TRH with time using static in vitro cultures.

The TSH response to repeated TRH stimulation declines in total
micrograms secreted per TRH pulse, but (after the first TRH pulse) shows

no decline in the secretion rate for the first two minutes of the
response. These data conflict with the theory presented by Connors,
et al. (1979) of a "depletable but replaceable pool of intracellular
TSH. (Connors suggested that TSH exists within the cell in two pools:
(1) a depletable pool that can be replenished relatively rapidly, and
(2) a more slowly secreted, depletion resistant pool. Connors reported
this after he noted a decrease in the amplitude of the initial peak
TSH response to TRH but no decrease in the lower, plateau level of TSH
secretion that is characteristic of ten or thirty-minute TRH stimula-
tion.) A comparison of the peak and total TSH responses to repeated
TRH stimulation is shown in Figures 9 and 10 and is given in Table III.
While the peak TSH response in column A remained fairly constant after
the first TRH pulse, the total ug TSH secreted per TRH pulse decreased
with each successive stimulation. This evidence conflicts with the
"depletion resistant pool" suggested by Connors. If the depletable
pool is replaced rapidly and the non-depletable pool is not affected
by repeated challenges as Connors suggests, then one would expect the
peak TSH responses and total TSH responses to parallel for the TRH
pulses in column A at one, two and three hours. The data presented
here do not support the proposed theory of a depletion resistant TSH
pool. Rather, the total TSH response to identical TRH stimulation de-
creases as the superfusion progresses.

Special consideration should be taken when calculating the hor-
mone responsiveness to secretagogues using the superfusion system. The
amplitude of the TSH response to a two-minute TRH stimulation is not
always indicative of the total TSH response. When looking at the peak

amplitude, one can readily see that peak height does not correlate directly to the amount of TSH secreted (Figure 11, Table III). For this reason, it is important to look very carefully at the entire TSH response peak and not draw conclusions from the peak amplitude only.

The involvement of shortloop feedback regulation of TSH secretion has not been investigated before using the superfusion system. Previous studies investigating possible TSH shortloop feedback have examined changes in the TRH content of the hypothalamus. Motta, et al. (1969) noted that bovine TSH, when administered to thyroidectomized rats, resulted in decreased hypothalamic TRH content as measured by bioassay. Roti, et al. (1978) on the other hand, detected no decrease in hypothalamic TRH content or pituitary TSH secretion when thyroidectomized rats were given the same amount of bovine TSH. Roti concluded that the shortloop feedback system reported for other pituitary hormones was probably not part of the regulatory system for TSH. The results of this current superfusion study indicate the TSH can inhibit TRH-mediated TSH secretion at the level of the pituitary thyrotroph. The apparent discrepancy between this study and those previously performed may be due to the fact that these experiment examined the effect of TSH directly upon the pituitary and that normal, not thyroidectomized, rats were used for this model system. I conclude that TSH, when administered during and throughout the time of expected TSH secretion, can attenuate such secretion and that this effect is mediated at the level of thyrotroph.

Cyclic AMP has been reported to be the second messenger involved in TSH secretion from anterior pituitary cells (Labrie, et al., 1978).

Results from this study do not give support to this role for cyclic
AMP. No TSH response was observed when cells were superfused with
3 mM dibutyryl cyclic AMP for two or ten minutes. These results are
in agreement with others using static cultures (Sundberg, et al.,
1968) and pituitary cell superfusion (Connors and Hedge, 1981). In
other static cultures, cyclic AMP was shown to elicit a TRH response
only after a three hour incubation with 1.0 or 0.5 mM dibutyryl cyclic
AMP (Leuschen, et al., 1978; Moriarty, et al., 1978). If dibutyryl
cyclic AMP is to be used to investigate TSH secretion in the super-
fusion system, a longer exposure time is suggested.

Somatostatin was effective in blocking the TRH-mediated release
of TSH from anterior pituitary cells while not affecting baseline
secretion. This observation is in agreement with previous reports
(Connors, et al., 1981; Drouin, et al., 1976). On the other hand,
when extract of the pituitary stalk median-eminence (SME) was presented
to the cells in the presence of somatostatin, no inhibition of TSH
secretion was noted. No analysis of the SME extract was done; therefore
it is not possible to determine the actual molar concentration of TRH
presented to the cells when superfused with 1.0 or 0.5 U SME/ml. The
0.5 U SME/ml dose did elicit a TSH response comparable to that elicited
by 10^{-8} M TRH (Tables III and IV). Another point to consider is the
unknown concentration of somatostatin in the SME extract. The median
eminence contains the highest concentration of somatostatin found in
the rat brain (Brownstein, et al., 1975; Kobayashi, et al., 1977).
Since the SME extract carries an undetermined amount of both TRH and
somatostatin, more refined experimentation is required before detailed

comparisons can be made between the effects of somatostatin on TRH-mediated and SME extract-mediated TSH secretion.

The TSH responsiveness to potassium observed using the super-fusion system are comparable to those reported by Vale and Guillemin (1967) in static cultures. As with repeated TRH stimulation, the TSH responses to repeated potassium stimulation decrease in magnitude. This decrease is detected both in the amplitude of the response as well as the total quantity of TSH secreted. For this reason, it is difficult to determine if the second, attenuated TSH response to potassium seen in Figure 15 is due to somatostatin inhibition or merely the naturally occurring decline in TSH responsiveness to potassium.

A final consideration when dealing with superfused pituitary cells is the availability of intracellular TSH for secretion. While some investigators have reported TSH responses after several days in static cell cultures (Dannies and Markell, 1980; Vale, et al., 1972), some of the cell preparations superfused in this study cease to secrete TSH after only eight to ten hours. This decrease in TSH responsiveness may be directly related to the continuous flow of fresh medium. Approximately 80% of the intracellular TSH is lost during the pre-incubation equilibration period, leaving only 20% available for secretion during the remainder of the superfusion experiment. Considering the effect of extracellular TSH on TSH secretion, it is likely that the tremendous loss of TSH from the cells during the first forty minutes of the pre-incubation period of the superfusion (Figure 7) does not occur in static cultures due to shortloop feedback inhibition. This could explain the difference in TSH responsiveness noted between static cultures and

superfused cells. Exhaustion of cellular TSH may very likely be a contributing factor to the decline in TSH responsiveness to secretagogues observed using the superfusion system.

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RADIOIMMUNOASSAY OF RAT TSH

Materials and Reagents:

1. 10 x 75 mm disposable borosilicate glass (or plastic) tubes
2. 0.01 M phosphosaline buffer, pH 7.6 with 1% bovine serum albumin (BSA)
3. 0.01 M phosphosaline buffer, pH 7.6 with 0.1% bovine serum albumin (BSA)
4. 0.01 M phosphosaline buffer, pH 7.6 with 3% normal rabbit serum and 0.05 M EDTA
5. Rat reference preparation, for standards, 10 ug/100 ul, stored frozen in 100 ul aliquots in 0.01 M phosphosaline buffer, pH 7.6 with 1% BSA
6. 125-I-TSH repurified within 24 hours of use; diluted to 10,000 cpm/100 ul with 0.1% BSA-PBS
7. TSH antiserum, stored frozen at 1:10 dilution with 0.01 M phosphosaline buffer-3% NRS - 0.05 M EDTA, pH 7.6. This is diluted to the appropriate titer for the assay (usually 1:10,000 f.d.) and stored in the refrigerator for up to one month.
8. Second antibody (goat or sheep anti-rabbit gamma globulin)

Assay Procedures:

1. Standards:
   Prepare 3 standard curves from 2.5 ng-1000 ng/tube using a micro-medic autodilutor. Dilute the thawed TSH reference preparation 1:10 with buffer prior to use for Stock A. Prepare two subsequent 1:10 dilutions for Stocks B and C. Use 100, 75, 50, and 25 ul of each stock solution for each standard curve.

2. Maximum Binding Tubes (Max or 100% or B_0 tubes):
   Prepare 4-10 Max tubes distributed in pairs throughout the assay. Add 0.2 ml 0.01 M PBS - 1% BSA to these tubes.

3. Blank Tubes:
   Prepare 4-10 blank tubes distributed in pairs (with the Max tubes) throughout the assay. Add 0.2 ml 0.01 M PBS - 1% BSA to these tubes.

4. Plasma Pools (Controls):
   Prepare 2-4 tubes containing 100 ul (more or less depending upon TSH content) and 100 ul of PBS buffer. If 4 tubes are prepared, 2 should be placed at the beginning of the unknown samples and 2 at the end of the samples.

5. Unknown Samples (Plasma, pituitary extract, incubation media, etc.):
    Remove clots and mix sample well. Pipette 0.2 ml (or less) into each assay tube. Add sufficient 1% BSA-PBS buffer to tube to bring volume up to 0.2 ml if less than 0.2 ml sample is used.

6. Total Count Tubes:
    Prepare 5-10 tubes. Contain no 1% BSA-PBS buffer.

7. Adding ^{125}I-TSH and TSH Antiserum:
    These two reagents are added simultaneously using 2 Micromedic autodilutor pumps.

    a. ^{125}I-TSH. Add 100 ul to each tube of assay.

    b. TSH antiserum. Add 200 ul to each tube of assay EXCEPT blank and total count tubes.

8. Blank Tubes:
    Receive 200 ul of the 3% NRS-0.05 M EDTA-PBS buffer.

9. Volume in all tubes except total count tubes should be 0.5 ml. Vortex, cover with Parafilm and incubate 24 hours at room temperature.

10. Second Antibody:
    Add sufficient antibody to maximally precipitate the antibody found rat TSH (usually 20-100 ul).

11. Vortex tubes, cover with Parafilm and incubate at $4^{o}C$ for 24 hours.

12. Centrifuge all tubes except total count tubes for 20 minutes at 1000 xg at $4^{o}C$.

13. Aspirate off the supernate immediately.

14. Count precipitate for 2 min. (or 10,000 counts) using automatic well type gamma counter.

TSH IODINATION PROCEDURE

Materials and Reagents:

1. 10 ml disposable plastic pipette for column
2. Glass bead, or glass wool
3. 30 collection tubes, 12 x 75 mm disposable glass
4. Sephadex G-50 (or G-75), in 0.01 M PBS
5. Bovine serum albumin (BSA), 2% and 5% in 0.01 M phosphosaline buffer
6. Phosphosaline buffer (PBS); 0.01 M, 0.50 M and 0.05 M, pH 7.6 (potassium or sodium)
7. Carrier-free Na I^{125} (NEN), 1.5 mCi
8. Chloramine T, 2.8 mg/ml in 0.05 M phosphosaline buffer, prepared fresh
9. Sodium metabisulfite, 2.4 mg/ml in 0.05 M PBS
10. NIAMD Rat TSH, 5 ug/25 ul in 0.01 M PBS, stored in reaction tube (10 x 75 mm disposable glass)
11. 0.5 M Phosphate buffer, pH 7.6

Preparation of Sephadex Column:

We generally prepare 2 columns so that there is a spare in case of an accident to one column or a bad iodination.

Swell Sephadex in 0.01 M PBS for 24 hr. ($\sim$1 g in 30 ml).

Decant to about 50:50 Sephadex to buffer and put under vacuum for about 30 min. to eliminate bubbles (or allow to reach room temperature without vacuum).

Cut off mouthpiece of column pipette, put in glass bead and fit tip with short section of rubber tubing with pinch clamp.

Fill column pipette with Pasteur pipette until bed volume is 7-10 ml.

Wash column with 10 ml of 0.01 M PBS which was previously put under vacuum for about 30 min. and drain meniscus to top of bed.

Coat Sephadex by adding 2 ml of 2% BSA and allowing to drain until meniscus is at top of bed.

Wash column with 10 ml 0.01 PBS and drain meniscus to approximately 1 ml above top of bed.

Label and coat the collecting tubes with 4 drops of 2% BSA/tube (only tubes 5-15 need BSA).

Check to see that all reagents and materials are prepared for the reaction, then drain meniscus to top of bed and clamp column.

Column is used once and discarded.

Labeling Procedure:

Make chloramine T and sodium metabisulfite immediately prior to use.
1. Thaw rat TSH (5 ug in 25 ul) in reaction vial.
2. Add 25 ul 0.5 M phosphate buffer to vial, mix gently.
3. Add 1.5 mC Na 125-I in 15 ul 0.5 M PBS, mix gently.
4. Add 20 ul of chloramine T (56 ug) and agitate gently for exactly 40 seconds.

5. Add 50 ul of sodium metabisulfite (120 ug), mix rapidly and apply reaction mixture to the Sephadex column.
6. Rinse vial with a few drops of 0.01 M PBS and add to top of column.
7. Drain meniscus to top of column and slowly begin adding 0.01 M PBS a few drops at a time, allowing meniscus to drain after each application. Do NOT ALLOW SEPHADEX TO BECOME DRY. Collect 8-9 drop fractions as they are eluted from the column. Add more PBS as needed. (Fill column once meniscus has drained several times.)
8. Count tubes to determine the location of the two peaks; one labeled hormone and one free 125-I.

APPENDIX II

PROTOCOL FOR PITUITARY CELL DISPERSION

<u>Preparatory Steps</u>: Have agitating water bath pre-heated to 37°C. Label four plastic disposable petri dishes, 1 - 4, and put 4 ml of Hanks HEPES buffer (pH 7.3) in each. Keep dishes heated to 37°C the entire time they are in use.

Cell Dispersion:

1. Sacrifice animals by sudden decapitation. Collect trunk blood in test tube if desired. Record the time of sacrifice of the first and last animal.
2. Quickly remove the pituitary gland and place in dish #1. Carefully remove the stalk median eminence and place in 0.1 N HCl on ice.
3. Wash pits through dishes 1, 2, and 3.
4. Remove posterior lobes and discard. Place an anterior lobe in dish #4.
5. With scissors or a razor blade, mince or dice the pits in a fifth siliconized glass watchglass or petri dish. Separate each pit into approximately twenty pieces.
6. Place all pieces into 5 ml of Hanks-HEPES buffer (H-H buffer) in a 15 ml conical centrifuge tube. Then wash dish #5 with 5 ml H-H buffer and collect in the same centrifuge tube.
7. Spin cells at 240 g for 10 minutes. Aspirate supernatant.
8. Resuspend pellet in 5 ml Collagenase solution. Agitate the cells in solution in a waterbath at 37°C for 30 min. Mix the cell suspension every 10 min. with a siliconized Pasteur pipet. Draw the cells into the pipet approximately 25 times the first two times and 100 times on the third and final mix.
9. Filter the cell suspension through nylon mesh (Nilex #102) into a second conical centrifuge tube. Wash the mesh with 5 ml H-H buffer. Spin the cells at 240 g for 20 min., check the supernatant for cells. If supernatant is clear, aspirate.
10. Wash cells in 5 ml H-H buffer and spin at 240 g for 20 min. Aspirate off supernatant.
11. Resuspend cells in 2 ml of buffer to be used in experiment. Test for cell number and viability.
12. Dilute the 2 ml cell suspension to desired cell concentration.

Equipment for Cell Dispersion:

4 small petri dishes
1 siliconized watch glass or petri dish
siliconized Pasteur pipets
curved tipped Pasteur pipet
nylon mesh
water bath
hemacytometer

microscope
adjustable pipet
15 ml conical centrifuge tubes
surgical tools:
    small curved forceps
    large curved forceps
    scissors
    scalpel
    Rongeur forceps
    razor blade (siliconized)

APPENDIX III

SUPERFUSION MEDIA COMPOSITION AND PREPARATION

Hanks-HEPES Buffer (H-H buffer): 500 ml

To 450 ml double distilled water, add the following reagents:

	G.M.W.	Conc. (mM)	gr./500 ml
NaCl	58.44	129.0	3.77
KCL	74.55	4.0	0.149
Na_2NPO_4	141.96	1.0	0.071
KH_2PO_4	136.09	0.6	0.041
$MgSO_4$	120.40	0.2	0.012
$MgCl_2$	203.23	0.5	0.051
$CaCl_2$	147.03	1.0	0.0735
glucose	180.16	6.0	0.540
$NaHCO_3$	84.01	15.0	0.630
HEPES (Sigma)	238.30	25.0	2.979

Adjust pH to 7.3 and Q.S. to 500 ml

Krebs-Ringer Bicarbonate Buffer (KRB):

KRB buffer is prepared with NaCl, KCl, $CaCl_2$, KH_2PO_4, and $MgSO_4$ solutions made up at 5 times the final concentration and stored at 4^OC.

Solution #	Conc. (mM)	5x Conc. (mM)
1. NaCl	154.0	770.0
2. KCl	154.0	770.0
3. $CaCl_2$	110.0	550.0
4. KH_2PO_4	154.0	770.0
5. $MgSO_4$	154.0	770.0

KRB buffer is made up fresh daily as follows: Solutions 1-5 which had been prepared previously were diluted at the ration 1:2:3:4:5 = 100:4:3:1:1. This solution was then diluted 1:5 in double distilled water to which was added 26 parts $NaHCO_3$ (154.0 mM). The KRB solution was adjusted to pH 7.3 after which glucose (11.0 mM) and bovine serum albumin (BSA 0.5 mg/ ml) were added. The KRB is maintained at 37^OC in a 95% O_2 - 5% CO_2 atmosphere for the duration of the experiment.

Diluting Fluid for Cell Count

Diluting fluid for the cell count procedure is made up as outlined by Davidson and Henery (1969) and modified by Hedge (personal communication) as follows:

1% w:v   crystal violet
1% v:v   glacial acetic acid
2 drops 5% phenol
100 ml double distilled water