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**DOPAMINERGIC INFLUENCES ON PROLACTIN SYNTHESIS AND
RELEASE FROM RAT ANTERIOR PITUITARY CULTURES**

The Ohio State University

PH.D.

1979

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DOPAMINERGIC INFLUENCES ON PROLACTIN SYNTHESIS AND RELEASE
FROM RAT ANTERIOR PITUITARY CULTURES

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of the Ohio State University

By

Kwang Chul Kim, B.S., M.Sc.

* * * * *

The Ohio State University

1979

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PREFACE

Life is a constant struggle against the tendency
to produce entropy. I am proud and grateful
that my life has been led to climb the mountains.

One winter morning,
in 1979

ACKNOWLEDGMENTS

I wish to express my sincere gratitude and appreciation to my adviser, Dr. Allan M. Burkman, for his thoughtful guidance throughout my entire Ph.D. training program. The execution and completion of this study would not have been possible without his patience, encouragement, and support. I wish to thank the reading committee for their constructive criticism, specially Drs. Dennis Feller and Norman Uretsky for their stimulating discussions and encouragement. Special acknowledgments are extended to Mr. John Hanson for his excellent electron microscopy and Mr. Judson Groshong for his generous help in my learning the details of the radioimmunoassay technique.

My most grateful appreciation is given to my parents for the guidance and sacrifice they gave me during the formative years of my life.

And last, but certainly not least, a special recognition must be given to my wife for her understanding and patience, but most of all, for her silent lessons of the life.

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Chapter I

Dopamine in the Hypothalamus: Its Relation to the Prolactin Inhibiting Factor (PIF)

Introduction

Prolactin (PRL) is one of several polypeptide hormones secreted by the anterior pituitary gland. It is released from lactotrophs (also called mammotrophs, alpha 2-acidophils, or PRL cells) which have been distinguished from other cell types within the pituitary by differential staining, electron microscopy and immunofluorescence techniques (Pasteels, 1972). The chemistry and biology of PRL have been reviewed in detail by Lewis (1975) and Cowie and Forsyth (1975).

The in vivo secretion of PRL by the anterior pituitary gland is normally inhibited by the hypothalamus. The pituitary gland secretes larger amounts of PRL when transplanted to areas remote from the hypothalamus (Everett, 1954), when incubated in vitro (Meites et al., 1961), or when lesions are placed in the median eminence region of the

hypothalamus (Chen et al., 1970).

Pasteels (1961) and Talwalker et al. (1963) were among the first to demonstrate that an acidic extract of hypothalamic tissue inhibited the in vitro secretion of PRL. The system of Talwalker et al. (1963), however, was refractory to a number of known neurological agents such as acetylcholine, epinephrine, histamine, substance P, oxytocin, and arginine or lysine vasopressin. They postulated that the hypothalamus contained an unidentified factor(s) that inhibits the in vivo synthesis and release of PRL. Hence, the term "prolactin inhibiting factor" (PIF) was given to that substance.

The chemical nature of PIF has been the subject of intense interest during the last decade. (See reviews by Nicoll, 1971; Meites and Clemens, 1972; MacLeod, 1976; Arimura and Schally, 1977; Kordon et al., 1977; Enjalbert et al., 1978; Flückiger, 1978; Tindal, 1978). At present, there are two different theories about the nature of PIF. One claims that PIF is dopamine (DA) (Shaar and Clemens, 1974; Gibbs and Neill, 1978), while the other maintains that PIF is a mixture of DA and some unknown hypothalamic peptide(s) (Greibrokk et al., 1975; Schally et al., 1976; Enjalbert et al., 1977).

Evidence has accumulated to support the view that DA is, indeed, involved in controlling plasma PRL levels in mammals. The major arguments are as follows:

1. The hypothalamus contains dopaminergic neurons (tuberoinfundubular dopamine fibers), the terminals of which form a dense network around the primary capillary plexus in the median eminence in rats (Fuxe, 1963; Hökfelt, 1967).

2. Significant quantities of DA can be detected in the hypophyseal portal blood of the rat (Ben-Jonathan et al., 1977).

3. DA inhibits PRL release both in vivo (Kamberi et al., 1971; Lu and Meites, 1972) and in vitro (Shaar and Clemens, 1974) in rats.

4. The inhibition of in vitro PRL secretion by DA in rats can be blocked by DA antagonists such as perphenazine and haloperidol (MacLeod and Lehmyer, 1974a).

5. The systemic injection of DA antagonist such as pimozide and haloperidol have been shown to increase plasma PRL levels in rats (Dickerman et al., 1972; Meites and Clemens, 1972).

On the other hand, other evidence seems to support the view that DA in the hypothalamus causes the release of PIF(s) from the median eminence, which then acts on the anterior pituitary together with DA to inhibit PRL secretion. These are:

1. Hypothalamic extracts contain peptide(s) as well as DA which exhibited PIF activity in the rat anterior pituitary organ culture (Greibrokk et al., 1975; Schally et al.,

1976; Enjalbert et al., 1977).

2. Anatomically, the median eminence of the hypothalamus contains peptidergic and other non-dopaminergic nerve terminals as well as dopaminergic ones (Brownstein, 1977).

3. Specific DA binding sites were demonstrated in the median eminence as well as the anterior pituitary (Cronin et al., 1978; Cronin and Weiner, 1979). Implants of pimozide, a dopaminergic receptor blocker, in the median eminence but not in the anterior basal hypothalamus increased plasma PRL level (Ojeda et al., 1974). Implantation of pimozide directly into the anterior pituitary showed smaller increases in plasma PRL than it did when implanted into the median eminence.

4. An inverse relationship was reported between DA levels in the hypophyseal portal blood and the serum PRL levels in pregnant, proestrous and estrous rats (Ben-Jonathan et al., 1977). However, this relationship did not exist in diestrous and male rats (Ben-Jonathan et al., 1977) which are known to have relatively low PRL levels. This seems to indicate that some endogenous principle(s) as well as DA may be involved in controlling PRL release in diestrous and male rats.

In general, the evidence supporting the second theory does not seem to be very strong, yet it still deserves attention. Deficiencies in experimental design made the results of these studies questionable and it is not

possible, at this time, to accept or dismiss the presence of a non-dopaminergic modulator of PRL release.

Studies by Cronin et al. (1978) and Cronin and Weiner (1979) regarding the existence of specific DA binding sites in the median eminence were criticized because of small sample size. It was previously shown by Brown et al. (1976) that high affinity receptors for [3 H]-dopamine and [3 H]-haloperidol were present in the rat anterior pituitary while none were detected in the rat basal hypothalamus. Thus, the presence of specific DA receptors in the median eminence seems to be somewhat uncertain. Even if the specific DA receptors do exist in the median eminence, they do not need to be associated with PIF secretion. It is equally likely that DA receptors in the median eminence may be on the neurons that secrete Luteinizing Hormone Releasing Hormone (Rotsztejn et al., 1977)

Ojeda et al. (1974) who implanted pimozide in different areas of the rat brain were not able to describe the exact drug concentrations that were at the sites of action following the implantations. Since pimozide was shown to inhibit PRL release in vitro at high concentrations (MacLeod and Lamberts, 1978), the results of pimozide implantations could be interpreted differently. Pimozide implanted in the median eminence may have diffused into the hypophyseal portal blood to act directly on the anterior pituitary at concentrations that block DA receptors. On

the other hand, pimozide implanted in the anterior basal hypothalamus may not have leaked into the portal blood at sufficient concentrations to block DA receptors in the anterior pituitary. Finally, pimozide implanted directly on the anterior pituitary may have reached so high a concentration that the PRL release was suppressed.

It was shown by Ben-Jonathan et al. (1977) that hypothalamic secretion of DA was quite low in both diestrous female and intact male rats, which are known to have much lower plasma PRL levels when compared to PRL levels during female proestrus. Estrogens are necessary for the rhythmic PRL surge which occurs during estrous cycle (Neill et al., 1971). They were shown to increase PRL release by acting mainly on the anterior pituitary (Eikenburg et al., 1977; Jacobi et al., 1977; Lieberman et al., 1978; Vician et al., 1979). Lower levels of plasma PRL in male rats could be explained by the low estrogen level as compared to female rats (Meites and Nicoll, 1966; Ojeda et al., 1977). Therefore, there is precedent for the belief that PRL secretion can be modulated by non-dopaminergic substances.

It has been shown by several laboratories (Greibrokk et al., 1975; Schally et al., 1976; Enjalbert et al., 1977) that hypothalamic extracts contain another PIF(s) besides DA. Since the term "PIF" was entirely based on the ability of hypothalamic extracts to inhibit PRL release

from the in vitro anterior pituitary organ culture, the concentrations of PIF or related substances in the hypothalamic extracts should have been carefully considered in order for the term to be physiologically meaningful. Concentrations of hypothalamic extracts used by these authors to demonstrate PRL inhibition were very high and seem to be more pharmacological than physiological.

The major objective of the studies described in this chapter is to reexamine, using pharmacological tools, whether DA can account for all the PIF activity of hypothalamic extracts using monolayer cell cultures of the rat anterior pituitary. The advantages of cell culture over organ culture methods, that have been used by others (e.g. Shaar and Clemens, 1974), will be described in the Discussion section.

The problem has been approached by examining the quantitative response of rat anterior pituitary cells in culture, to (a) varying concentrations of dopaminergic drugs in order to characterize the PRL secretory responses, (b) concentrations of hypothalamic and cortical extracts, and (c) various DA blockers in the presence and absence of hypothalamic extracts and DA.

Materials and Methods

Animals

Mature male Sprague-Dawley rats (Harlan Industries, Cumberland, Indiana) weighing 250-300 g, were used as pituitary donors. Animals were maintained in a light-controlled room (lights on from 0600 to 1800 hr), and allowed water and Purina Lab Chow ad libitum.

Culture preparation

The details of the procedures can be found in Kahn (1973) and Hilfer (1973). Following decapitation of the animal, the anterior pituitary was removed aseptically, separated from the posterior lobe, washed three times in sterile culture medium^a, minced into 2 mm pieces with a sterile scalpel and incubated in 7.5 ml of Ca^{++} and Mg^{++} free Hank's base^a containing 0.47 % collagenase^a for 50 min, with gentle stirring at 37° C in a 95 % O_2 - 5 % CO_2 atmosphere. The dissociated cell suspension was centrifuged twice at 67 x g for 10 min at 4° C and washed each time with the Hank's base in order to remove collagenase. The pellet was resuspended in culture medium (20 ml/pituitary) and distributed into sterile 16 x 10 mm CostarTM tissue culture wells (0.5 ml/well) while stirring. These conditions were found to be the most appropriate for the

^a See Appendix A.

present experiments. The cell density of inoculum was 0.5 to 1.0×10^5 cells/ml medium. All the subsequent incubations were conducted at $37.0 \pm 0.5^\circ\text{C}$ and saturated humidity. The pH of the medium was maintained at 7.3 ± 0.1 by controlling the ratio of CO_2 to air flowing into the incubator. An atmosphere of 1 - 1.5 % CO_2 was adequate.

Culture medium^a

The culture medium consisted of complete Minimum Essential Medium (EAGLE) supplemented with 0.1 mM Non-essential amino acids, 1 mM sodium pyruvate, 40 mg gentamicin sulfate/l medium, and 10 % fetal bovine serum.

Experimental procedures

Following the inoculation, the culture medium (1 ml/well) was changed every three days until the day of experimentation. All the experiments were done during the pre-confluent monolayer period. Each experiment consisted of a 4 - 7 hr preincubation followed by a 4 - 6 hr treatment period. At the end of preincubation, an aliquot of 0.1 ml of each culture medium was withdrawn and replaced with fresh medium. The preincubation aliquot was assayed for PRL for the purpose of establishing the basal PRL producing activity of the cells. All drugs and vehicle solvents

^a See Appendix A.

were subsequently added in a volume of 10 μ l to culture wells containing 1 ml medium, while hypothalamic and cortical extracts were added in a volume of 0.1 ml into culture wells containing 0.9 ml medium. Both preincubation and treatment period samples were diluted with 0.1 % bovine serum albumin-phosphate buffer^a and immediately frozen at -20° C until assayed.

Assays

The amounts of the secreted PRL were measured using radioimmunoassay (RIA). RIAs were performed as described in the National Institute of Arthritis, Metabolism, and Digestive Disease (NIAMDD) rat pituitary hormone distribution program^a. Kits for the rat PRL assays were generously provided by that institute. NIAMDD rat PRL-I-3 was iodinated^a with Na¹²⁵I and purified^a, and the labeled PRL was repurified^a immediately before each RIA. Dextran-coated charcoal was prepared and used as described in Herbert et al. (1965) in order to remove the free PRL. All samples were assayed in duplicate. PRL values were expressed in terms of the reference preparation, NIAMDD rat PRL-RP-1, and calculated either directly from a standard curve such as Fig. 1. or from a computer program based on the standard curve^a.

^a See Appendix B.

Figure 1. A representative curve of a rat prolactin radioimmunoassay. Each plotted value is the mean of triplicate determinations.

Statistics

Each culture well yields a pair of data, the amount of PRL release during the preincubation period (preincubation value) and that during the treatment period (treatment value). The ratio of treatment period value to preincubation period value was calculated for each experimental well. The mean of the ratios from each group was compared with each other to assess the significant difference between groups. Statistical analysis included (a) variance homogeneity tests and (b) Student's unpaired t-tests for samples with equal or unequal variance. In expressing data, the mean of the ratios from the control group was set at 100 %, and means of the ratios of treatment groups were expressed as % control.

Drugs

Dopamine HCl (Sigma) was prepared in 0.9 % (w/v) saline containing 0.05 % (w/v) sodium metabisulfite. Haloperidol (Sandoz) was dissolved in 0.3 % (w/v) tartaric acid and further diluted with the saline-metabisulfite vehicle. Isomers of butaclamol HCl (Ayerst) were dissolved in n-isopropyl alcohol and further diluted with the saline-metabisulfite vehicle. Tartaric acid and alcohol contents were less than 0.003 % and 1 % of the final solution, respectively. Control groups were given the same amounts of the solvent and vehicle as treatment groups.

Preparation of hypothalamic extracts

Hypothalami were removed as described in Glowinski and Iversen (1966) and were immediately frozen at -20°C and stored, usually several days, before experimentation. Extraction was performed according to the modification of Shaar and Clemens (1974). Immediately before treatment, 10 to 20 frozen hypothalami were thawed and homogenized as a pool with a glass Ten-Broeck homogenizer containing chilled 0.1 N HCl (0.3 ml/hypothalamus) and centrifuged at $30,000 \times g$ for 20 min at 4°C . One rat hypothalamus weighed about 100 mg. The supernatant was decanted and neutralized to pH 7.0 with 2 N NaOH. The neutralized solution was recentrifuged at $3,000 \times g$ for 20 min at 4°C . The supernatant was decanted and partially evaporated to a desired volume under reduced pressure while mechanically shaking in a cold bath (Evapo-MixTM, Buchler Instruments). Extracts were diluted, if necessary, to a desired concentration. Concentrations of hypothalamic extracts were expressed as number of hypothalamic equivalents in 0.1 ml of the final hypothalamic extract solution. Thus, a hypothalamic extract equal to one hypothalamic equivalent means that 0.1 ml of the hypothalamic extract solution contains the products of one hypothalamus.

Preparation of cortical extracts

During removal of hypothalami from rats, cortical slices (mainly frontal cortex) were collected, frozen at -20°C , and stored not more than a week. Cortical slices of the same weight as hypothalami were subjected to the same preparative procedures as were the hypothalami.

Electron microscopy

Treated and untreated cells were examined for morphological changes using light (phase), scanning and transmission microscopy.

Scanning electron microscopy - Coverslips with adhering cells were fixed in 4 % glutaraldehyde buffered with Sorenson's phosphate buffer. The cells were dehydrated in ethanol, critical point dried, and vacuum coated with gold. The coated slips were examined with a Hitachi S500 electron microscope.

Transmission electron microscopy - Monolayers were dispersed using 0.25 % (w/v) trypsin and 0.02 % (w/v) EDTA prepared in Ca^{++} and Mg^{++} free Hank's base. They were centrifuged at $70 \times g$ for 15 min at 4°C , and the resulting pellets were postfixed in OsO_4 , dehydrated and embedded in Epon 812 resin. Ultrathin sections were cut using a diamond knife, stained with uranyl acetate and lead citrate, and examined with a Zeiss EM 95-2.

Results and Discussion

Rat anterior pituitary monolayer cell culture

Cell culture is a very useful technique for studying the behavior of endocrine cells in a controlled environment. The major advantages of cell culture over organ culture methods include (a) much smaller variations in basal activity among individual culture dishes, (b) absence of diffusion barrier to O_2 , nutrients and drugs, (c) larger number of groups of viable cells that can be studied during a given incubation period, and (d) greater economy in terms of both preparation time and animals.

After dispersion by collagenase treatment, all the pituitary cells exhibited a spherical shape. During the first 3 days in culture, a significant portion of them were already tightly attached to the bottom surface of the dishes. The glandular cells at this stage developed a less regular shape and contained clearly distinct secretory granules around the periphery of the cells as visualized by phase contrast light microscopy in Plate I. They were grouped in small irregular clusters.

After the fifth day, fibroblastic cells appeared more abundantly and thereafter became increasingly numerous. These cells were more elongated and transparent than the glandular cells and had less distinctive outlines.

After the seventh day, the overall appearance remained almost unchanged. Most of experiments were performed during the second week following inoculation. During the second month, several sheets of fibroblastic cells were superimposed onto the dish and the glandular cells became also confluent.

Rates of PRL release after initial culture

The rates of PRL release into the medium following inoculation are shown in Table 1. They increased almost linearly after five days of culture. There is no clear evidence that lactotrophs proliferate during the monolayer cell culture. However, this continuous increase in PRL secretion rates during culture may be due to the increase in number as well as in secretory activity of lactotrophs, because (a) the number of glandular cells in culture increased, finally reaching a state of confluency, and (b) lactotrophs become dominant in appearance during the second month of the monolayer cell culture (Tixier-Vidal, 1975).

PRL release during a short-term incubation and its inhibition by DA

During incubation with fresh medium, PRL is released almost linearly for at least a 6 hr period. Table 2 shows that DA at 10^{-5} M concentration can inhibit PRL release at a consistent degree between 2 and 5 hr treatment period.

TABLE 1
Rates of PRL Release During
Anterior Pituitary Cell Culture in Rats

Days following inoculation	PRL (ng/hr/well)
5	14.0 ± 0.4 (3)
8	36.3 ± 2.9 (2)
10	41.3 ± 0.5 (3)
13	62.9 ± 0.9 (3)
15	77.1 ± 4.6 (3)
19	115.6 ± 5.1 (3)
22	140.9 ± 11.1 (3)

Following inoculation of cells into culture wells, the culture media were replenished every three days until the day of sampling. Before sampling, cells were washed and incubated in fresh medium for 4-7 hr. The amount of PRL secreted during this period was measured using RIA. Each value represents a mean ± SEM and numbers in parentheses represent the number of culture wells used in each group.

TABLE 2
Time Course of PRL Release
in Presence and Absence of 10^{-5} M DA

Treatment	Cumulative PRL release (TP/PI)			
	1 hr	2 hr	4 hr	5 hr
Control	17.5±1.5	39.6±2.7	69.5±6.7	92.3±5.7
DA (10^{-5} M)	7.7±0.2	11.7±0.5	20.5±0.4	27.0±0.7
DA/Control (%)	44.0±1.1	29.5±1.3	29.5±0.6	29.3±0.8

Fifteen day old culture was used. PRL release was expressed as a ratio(in percent) of the amount of PRL released during a given hour of treatment period (TP) to that released during a six hour preincubation (PI) period. Each value represents a mean ± SEM of three wells.

Quantitative effects of DA on PRL release

DA inhibited PRL release in a dose-dependent fashion as can be seen in Fig. 2. The median effective dose (mean $\pm$ SEM) of DA in inhibiting PRL secretion was $(2.0 \pm 0.2) \times 10^{-7}M$ ($n=4$). The maximal inhibition ranged from 70 to 90 % of the control and that was produced by $10^{-5}M$ DA. The further increase in concentrations of DA did not enhance the degree of inhibition. These data suggest the existence of a DA-independent release mechanism.

On occasion, a stimulatory effect was observed by DA at $10^{-9}M$ concentration (Fig. 3). This biphasic response, i.e., stimulation of PRL release at low concentrations and inhibition at high concentrations, was previously reported to occur in the rat anterior pituitary organ culture, when norepinephrine and epinephrine (Koch et al., 1970), and hypothalamic extracts (Nicoll et al., 1970) were added. Shin (1978), using DA in a cell culture system, made a similar observation. Neither the reason for the inconsistency nor details of the biphasic response in in vitro anterior pituitary culture are known at present.

Effects of haloperidol on PRL release

Haloperidol at low concentrations showed no significant effects on PRL release as compared with control (Table 3). However, $10^{-5}M$ haloperidol inhibited PRL release by about 50 % while more than two fold stimulation

Figure 2. Effect of DA on PRL release. PRL release from each group was expressed as a ratio (in %) of the treatment TP/PI to the control TP/PI. Each point is a mean $\pm$ SEM of 6 twelve day old culture wells. Treatment period was 5 hr.

Figure 3. The biphasic effect of DA on PRL release. PRL release from each group was expressed as a ratio (in %) of the treatment TP/PI to the control TP/PI. Each point is a mean $\pm$ SEM of 5 seven day old culture wells. Treatment period was 6 hr.

was shown at 10^{-4} M concentration (Table 3).

(+)Butaclamol, another DA antagonist, also suppressed PRL release at 10^{-5} M concentration (Table 5). Thus, at high concentrations, DA antagonists such as haloperidol and (+)butaclamol could be partial DA agonists on the lactotroph DA receptors.

Recently, MacLeod and Lamberts (1978) reported the similar findings that, at high concentrations, pimozide and haloperidol, could suppress the release of newly synthesized PRL in the rat anterior pituitary organ culture. This inhibitory effect by the DA antagonists could be either due to their partial agonistic property or through a non-dopaminergic inhibitory effect. However, the inhibition does not seem to be due to their partial agonistic property for the following reasons:

1. This inhibitory activity could not be blocked even with high concentrations of neuroleptics devoid of any inhibitory activity in the anterior pituitary cell culture (Denef and Follebouckt, 1978).

2. Both (+) and (-)butaclamol which are considerably different in DA antagonistic potency were equipotent in inhibiting PRL release in the rat anterior pituitary cell culture (West and Dannies, 1979).

3. The concentrations of haloperidol (10^{-5} M and 10^{-4} M) and (+)butaclamol (10^{-5} M) which suppressed PRL release caused some irreversible toxicity on anterior pituitary

TABLE 3
Effects of Haloperidol on PRL Release

Dose	% Control ^a	Statistics ^b
Control	100.0 ± 2.5	
10 ⁻¹⁰ M	107.5 ± 9.0	NS(p>0.05)
10 ⁻⁹ M	99.9 ± 7.7	"
10 ⁻⁸ M	111.5 ± 11.3	"
10 ⁻⁷ M	103.2 ± 2.2	"
10 ⁻⁶ M	100.1 ± 8.2	"
10 ⁻⁵ M	53.0 ± 3.8	S(p<0.001)
10 ⁻⁴ M	261.2 ± 7.3	"

^a PRL release from each group was expressed as a ratio(in %) of the treatment TP/PI to the control TP/PI. Each value represents a mean ± SEM of 3 nineteen day old culture wells. The treatment period was 6 hr.

^b Comparison with control group.

cells as can be seen in Plates I to VIII. This type of cytotoxicity was neither caused nor reversed by very high concentrations (up to $10^{-3}M$) of DA.

The toxicity was observed in the following sequences:

1. During 5-7 hr following treatment, PRL release was significantly suppressed and the secretory granules increased in number, density and prominence.

2. After 1-3 days following treatment, cell membranes of both glandular and fibroblastic cells were severely damaged and number of secretory granules and intact cells decreased dramatically.

The two fold increase in PRL secretion during $10^{-4}M$ haloperidol treatment (Table 3) seems to have resulted from the membrane damage of lactotrophs. Although the mechanism of haloperidol-induced cytotoxicity in the rat anterior pituitary cells cannot be explained clearly at present, it does not seem to be mediated through dopaminergic mechanism, because (a) high concentrations of DA (up to $10^{-3}M$) did not cause the cytotoxicity in these culture system, and (b) the pretreatment of high concentrations of DA could not prevent the haloperidol-induced cytotoxicity.

Plates I and II. Anterior pituitary cells in monolayer culture. Twelve day old culture was randomly divided into two groups, three culture dishes for each group. The dishes were treated with either $10^{-4}M$ haloperidol or its vehicle (0.01 % tartaric acid) for 9 hr and washed with fresh medium, immediately and every three days afterwards. Seven days following treatment, cells were fixed with formalin, stained with Wright's stain and observed under a phase contrast light microscope. Two types of cells, glandular and fibroblastic, are seen in the field. In control group (Plate I), the glandular cells have irregular shapes with mostly centered distinct nuclei and variable numbers of secretory granules, while fibroblastic cells are elongated, stretching between clusters of glandular cells and look transparent with elongated nuclei. In the drug-treated group (plate II), glandular cells markedly decreased in number due mainly to deaths, and cell debris are seen. Many fibroblastic cells look thinner and entangled. X 433.

Plate I

Plate II

Plates III and IV. Scanning electron micrographs of anterior pituitary cells. Eighteen day old culture was treated with either haloperidol ($10^{-4}M$) or its vehicle for 9 hr, and immediately processed for scanning electron microscopy as described in the section of Materials and Methods. In control group (Plate III), glandular cells show many spines projected from the cell surface and a fibroblastic cell shows a smoother cell surface. In the drug-treated group (Plate IV), a glandular cell shows not only lack of projections but also very severely damaged cell membrane, while a fibroblastic cell shows a very irregular cell surface. Debris seen on the surface of the fibroblastic cell seem to have originated from damaged glandular cells. X 3,200

Plate III

Plate IV

Plates V and VI. Transmission electron micrographs of anterior pituitary cells. Twenty day old monolayer cell culture was treated with either haloperidol ($10^{-4}M$) or its vehicle, and processed for transmission electron microscopy as described in the section of Materials and Methods. In control group (Plate V), a glandular cell contains many secretory granules and rich rough endoplasmic reticulum, while a fibroblastic cell (left half of the field) has an elongated nucleus and a smooth cell membrane. In the drug-treated group (Plate VI), a glandular cell (right half) shows almost no secretory granules and damaged cell membrane, while a fibroblastic cell (left half) shows irregular cell membrane. X 6,300.

Plate V

Plate VI

Plates VII and VIII. A part of an anterior pituitary glandular cell. The origin and treatment of cells used in these plates are the same as described in Plates V and VI. In control group (Plate VII), many secretory granules of various size and a regular cell membrane are seen, while, in the drug-treated group (Plate VIII), few secretory granules and severely damaged cell membrane can be seen. X 24,500.

Plate VII

Plate VIII

Specific DA receptors on rat anterior pituitary lactotrophs

The inhibitory effect of DA on PRL release could be blocked by DA antagonists such as haloperidol (Table 4) and (+)butaclamol (Table 5), but not by (-)butaclamol (Table 5). Mowles et al. (1978) also reported the antagonistic relationship between DA and haloperidol in the rat anterior pituitary cell culture. Butaclamol has been shown to be stereospecific on rat anterior pituitary DA receptors both in vivo (Meltzer et al., 1977) and in vitro (Caron et al., 1978).

Haloperidol at 10^{-7} M concentration was able to reverse all the effects of 10^{-5} M DA. However, 10^{-5} M haloperidol could not reverse the effect of 10^{-5} M DA, but showed about 60 % inhibition of PRL release. This inhibitory effect of 10^{-5} M haloperidol (Table 4) seems to be due to its non-dopaminergic activity as already discussed in the previous section.

One of the most useful pharmacological tools used in identifying the specificity of receptors is a Schild plot (Arunlakshana and Schild, 1959). A Schild plot of the relationship between DA and (+)butaclamol in anterior pituitary PRL release showed that pa_2 of (+)butaclamol was 8.9, as can be seen in Fig. 4. This value is in good agreement with IC₅₀'s or K_i's of (+)butaclamol obtained from binding studies (Beld et al., 1978; Caron et al., 1978; Creese et al., 1978; Titeler et al., 1978).

TABLE 4
Reversal of Effects of 10^{-5} M DA by Haloperidol

Treatments	% Control ^a	Statistics ^b
HC + DAC	100.0 ± 5.2 (6)	
HC + 10^{-5} M DA	13.3 ± 1.1 (3)	S(p<0.001)
10^{-9} M Hal + "	18.3 ± 0.9 (3)	"
10^{-8} M " + "	56.7 ± 1.0 (3)	"
10^{-7} M " + "	93.1 ± 4.3 (3)	NS(p>0.05)
10^{-6} M " + "	110.9 ± 4.5 (3)	"
10^{-5} M " + "	39.4 ± 0.7 (3)	S(p<0.001)
10^{-5} M " + DAC	53.0 ± 3.8 (3)	"

a,b See TABLE 3.

Eleven day old culture was used and the treatment period was 6 hr. DAC represents 0.9% saline containing 0.05% sodium metabisulfite used for DA carrier, while HC represents 0.003% tartaric acid in the saline-metabisulfite solution used for haloperidol carrier.

TABLE 5
Reversal of Effects of 10^{-5} M DA by (+)Butaclamol

Treatments	% Control ^a	Statistics ^b
BC + DAC	100.0 ± 3.9	
BC + 10^{-5} M DA	31.6 ± 7.3	S(p<0.001)
10^{-10} M (+)B + "	28.3 ± 7.5	"
10^{-9} " + "	24.5 ± 3.0	"
10^{-8} " + "	36.1 ± 2.7	"
10^{-7} " + "	49.1 ± 2.3	"
10^{-6} " + "	91.5 ± 0.8	NS(p>0.05)
10^{-5} M (+)B + DAC	61.3 ± 7.9	S(p<0.001)
10^{-6} M (-)B + 10^{-5} M DA	38.4 ± 5.2	NS(p>0.05) ^c

^{a, b} See TABLE 3. n=3.

^c Comparison with 10^{-5} M DA + BC treated group.

Six day old culture was used and the treatment period was 5 hr. DAC represents 0.9% saline containing 0.05% sodium metabisulfite used for DA carrier, while BC represents 0.01% n-isopropyl alcohol in the DAC used for butaclamol carrier.

Figure 4. Schild plot of (+)butaclamol against DA from their antagonistic relationship in PRL release in the rat anterior pituitary cell culture.

In light of the aforementioned data, it is clearly seen that the rat anterior pituitary cells in culture contain stereospecific DA receptors, stimulation of which causes the inhibition of PRL secretion in a dose-dependent fashion.

During the last five years, there has been some confusion about the classification of DA receptors (Cools and Van Rossum, 1975; Galea Debono et al., 1976; Goldberg et al., 1978; Titeler et al., 1978). At present, two classes of DA receptors are defined according to their association with, or independence from, a DA-sensitive adenylyl cyclase. DA receptors unrelated to adenylyl cyclase are designated as type alpha, while those linked to adenylyl cyclase are designated type beta (Kebabian, 1978). The dopaminergic ergot derivatives (lisuride, lergotrile and 2-bromo-alpha-ergocryptine) and their antagonists (molindone, sulpiride and metoclopramide) are relatively specific for alpha DA receptor. Other agonists (e.g. apomorphine and DA) and antagonists (e.g. antipsychotic phenothiazines and butyrophenones) affect both classes of receptors.

These types of DA receptors can also be distinguished by their binding affinity for DA drugs. The beta DA receptor is stimulated by micromolar concentrations of DA (Miller et al., 1974). In contrast, alpha DA receptors are stimulated by nanomolar concentrations of DA (Miller et al., 1974).

The anterior pituitary DA receptors responsible for control of PRL secretion are sensitive to alpha as well as beta drugs (Schmidt and Hill, 1977) with nanomolar affinity (Caron et al., 1978). These receptors may not be associated with adenylyl cyclase (Schmidt and Hill, 1977; Mowles et al., 1978). However, Ahn et al. (1979) recently reported the presence of DA receptors coupled to adenylyl cyclase in the rat anterior pituitary with micromolar affinity for DA. Guanosine triphosphate seems to regulate these DA receptors (Sibley and Creese, 1979). The physiological significance of these adenylyl cyclase coupled DA receptors are not known.

Effects of hypothalamic extracts on PRL release

Hypothalamic extracts were shown to suppress PRL release at 0.2 hypothalamic equivalents or higher concentrations (Table 6). Cortical extracts, which were used as control for hypothalamic extracts, also could inhibit PRL release but only at concentrations equal to or higher than one hypothalamic equivalent (Table 7). The inhibitory effect of cortical extracts was not blocked by $2 \times 10^{-7}M$ haloperidol, which was strong enough to block the even greater inhibition by DA.

Since almost all the studies designed to discover the active principle(s) in hypothalamic extracts which are responsible for the PIF activity have used concentrations much

TABLE 6
Effects of Hypothalamic Extracts on PRL Release

Treatments	% Control ^a	Statistics ^b
HE (2 HP) ^c	29.0 ± 0.6	S(p<0.01)
" (1 ")	39.1 ± 2.8	"
" (0.6 ")	67.2 ± 3.3	"
" (0.2 ")	78.8 ± 4.8	"
" (0.06 ")	93.6 ± 4.0	NS(p>0.05)
" (0.02 ")	95.5 ± 6.7	"
" (0.006 ")	94.1 ± 6.9	"

^a Each value represents a mean ± SEM of three wells. These values were obtained from several different experiments. Thus, each % control value was calculated from its own control value.

^b Comparison was made also with its own control. See TABLE 3 for details.

^c Hypothalamic equivalents (HP)
Hypothalamic extracts (HE)

TABLE 7
Effects of Cortical Extracts on PRL Release

Treatments	% Control ^a	Statistics ^b
Medium	100.0 ± 3.4	
CE (0.5 HP) ^d	86.4 ± 6.0	NS(p>0.05)
CE (1 HP)	56.8 ± 1.8	S(p<0.01)
Hal(2x10 ⁻⁷ M) + CE (1 HP)	57.1 ± 3.0	NS(p>0.05)
Hal(2x10 ⁻⁷ M)	106.2 ± 6.3	

^{a, b} See TABLE 3. n=3.

^c Comparison with CE (1 HP) treated group.

^d Cortical extracts (CE)
Hypothalamic equivalents (HP)

Fourteen day old culture was used and the treatment period was 5 hr.

higher than 0.5 hypothalamic equivalents per 1 ml culture medium, their results must be reconsidered.

Thus, in the present experiments, hypothalamic extracts of 0.5 hypothalamic equivalents whose cortical extracts counterpart did not show any inhibition was used to see whether the effect could be fully explained by dopaminergic mechanism. Hypothalamic extracts of 0.5 hypothalamic equivalents inhibited PRL release by about 40 % (Table 9).

In approaching this problem, two questions were asked:

1. Can all the PIF activity of hypothalamic extracts be blocked by specific DA antagonists ?
2. Is the inhibitory effect of hypothalamic extracts on PRL release additive to that of DA ?

Effects of hypothalamic extracts on PRL release in relation to DA

If the inhibitory effect of hypothalamic extracts is entirely due to DA contained in hypothalamic extracts, all the PIF activity of hypothalamic extracts should be reversed by DA antagonists such as haloperidol and (+)butaclamol. DA has been shown to be stable throughout the hypothalamic extracts preparation procedures (Table 8).

Table 9 shows that the inhibitory effect of hypothalamic extracts of 0.5 hypothalamic equivalents could be completely blocked by either 2×10^{-7} M haloperidol or

TABLE 8
Stability of DA During
the Preparation of Hypothalamic Extracts

Treatments	% Control ^a	Statistics ^b
1. Medium	100.0 ± 0.9	
2. DA (10 ⁻⁵ M)	26.0 ± 2.7	
3. HE (0.2 HP) ^d	84.0 ± 2.7	S(p<0.05)(1+3) ^c
4. DAE (10 ⁻⁵ M) ^e	29.3 ± 3.6	NS(p>0.05)(2+4)
5. (H + DA)E ^f	41.6 ± 5.4	S(p<0.05)(2+5)

^{a,b} See TABLE 3. n=3.

^c Treatment groups for comparison.

^d Hypothalamic extracts (HE)
Hypothalamic equivalents (HP)

^e DA subjected to HE preparation procedures.

^f HE in which DA was added at the beginning of HE preparation.

Eighteen day old culture was used and the treatment period was 5 hr.

TABLE 9
Effects of DA Antagonists on the Ability of
Hypothalamic Extracts to Inhibit PRL Release

Treatments	% Control ^a	Statistics ^b
Medium	100.0 ± 3.4	
HE (0.5 HP) ^c	59.9 ± 5.3	S(p<0.01)
Hal(2x10 ⁻⁷ M)+HE(0.5HP)	89.7 ± 6.8	NS(p>0.05)
Hal(")	98.5 ± 3.5	
(+)But(10 ⁻⁶ M)+HE(0.5HP)	84.6 ± 6.1	NS(p>0.05)
(+)But(")	90.2 ± 13.9	
(-)But(10 ⁻⁶ M)+HE(0.5HP)	68.1 ± 0.6	S(p<0.05)
(-)But(")	87.6 ± 4.8	

^{a, b} See TABLE 3. n=3.
Comparison was made with its own control which
does not contain HE.

^c Hypothalamic extracts (HE)
hypothalamic equivalents (HP)

Fourteen day old culture was used and the treatment
period was 5 hr.

10^{-6} M (+)butaclamol, but not by 10^{-6} M (-)butaclamol. The concentrations of haloperidol and (+)butaclamol used did not cause the inhibition of PRL release but were enough to completely reverse the much greater inhibition by 10^{-6} M DA. This result indicates that the PIF activity of hypothalamic extracts is due to dopaminergic principle(s) in the extracts.

On the other hand, if hypothalamic extracts contain a principle(s) which inhibits PRL release through non-dopaminergic mechanism, the combination of DA and hypothalamic extracts would show the greater inhibition than DA alone. Table 10 shows that the combination of hypothalamic extracts and DA did not cause any greater inhibition than DA alone. This can serve as another evidence that the PIF activity of hypothalamic extracts can be explained by a dopaminergic mechanism.

However, the combination of hypothalamic extracts and DA showed a net inhibitory effect lower than DA alone, and the net effect was completely reversed by 2×10^{-7} M haloperidol (Table 10). The partial reduction of the effect of DA on PRL release by hypothalamic extracts had been observed previously (Table 8). This result suggests that hypothalamic extracts contain a principle(s) which behaves as a partial DA agonist.

The hypothalamus is rich in norepinephrine and epinephrine as well as DA (Cuello et al., 1973).

TABLE 10
Effects of Combination of Hypothalamic
and Cortical Extracts with DA on PRL Release

Treatments	% Control ^a	
1. Medium	100.0 ± 2.3	
2. HE(0.5 HP) ^d	59.9 ± 5.3	
3. DA(10 ⁻⁶ M)	30.2 ± 0.7	
4. DA(10 ⁻⁶ M)+HE(0.5HP)	43.3 ± 2.5	S(p<0.01)(3+4) ^c
5. DA(10 ⁻⁶ M)+CE(0.5HP) ^e	33.1 ± 2.0	NS(p>0.05)(3+5)
6. DA(10 ⁻⁶ M)+HE(0.5HP) +Hal(2x10 ⁻⁷ M)	110.1 ± 8.4	NS(p>0.05)(1+6)
7. DA(10 ⁻⁶ M)+Hal(2x10 ⁻⁷ M)	97.3 ± 7.1	
8. DAC +Hal(2x10 ⁻⁷ M) ^f	98.3 ± 0.1	

^{a,b} See TABLE 3. n=3.

^c Treatment groups for comparison.

^d Hypothalamic extracts (HE)
Hypothalamic equivalents (HP)

^e Cortical extracts (CE)

^f DAC represents 0.9% saline containing 0.05% sodium metabisulfite used for DA carrier.

Fourteen day old culture was used and the treatment period was 5 hr.

The median eminence of the hypothalamus contains terminals of ventral noradrenergic neurons as well as tuberoinfundibular DA neurons (Fuxe et al., 1963; Björklund et al., 1973). All these catecholamines can inhibit PRL release in the rat anterior pituitary organ culture, their potency being 1 : 20 : 50 for epinephrine : norepinephrine : DA, respectively (Shaar and Clemens, 1974).

Several lines of evidence seem to indicate that norepinephrine and epinephrine possess the dopaminergic property:

1. The administration of these compounds into the supersensitive caudate nucleus caused vigorous long-lasting dose-dependent contralateral turning in rats (Setler et al., 1978).

2. They activate DA sensitive adenylyl cyclase in the rat caudate (Miller et al., 1974).

3. They are very potent in displacing ^3H -dopamine, ^3H -ADTN^a and ^3H -dihydroergocryptine from the calf striatum (Burt et al., 1975), the rat striatum (Creese et al., 1978) and the bovine anterior pituitary gland (Labrie et al., 1978), respectively.

4. They inhibited PRL secretion in in vitro organ culture (Shaar and Clemens, 1974). This inhibitory effect was completely reversed by perphenazine, a DA antagonist (Arimura and Schally, 1977).

^a 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene

Thus, the inhibitory effect of both norepinephrine and epinephrine on PRL release in the rat anterior pituitary cell culture seems to be mediated through specific DA receptors.

In summary, the PIF activity of hypothalamic extracts can not be accounted for by DA alone, but seems to be due to the combination of DA, norepinephrine and epinephrine, all of which act on the rat anterior pituitary DA receptors to inhibit PRL release. The present results seem to support the view that DA itself is a physiological PIF, since DA but neither norepinephrine nor epinephrine is detectable in the hypophyseal portal blood in normal rats (Ben-Jonathan et al., 1977) and DA levels in the hypophyseal stalk blood in the rat are sufficient to inhibit PRL secretion in vitro (Gibbs and Neill, 1978).

Finally, it is necessary, before DA is conclusively accepted as a physiological PIF, to show the good correlation between DA levels in the hypophyseal portal blood and the degree of inhibition of PRL release from the anterior pituitary, under a variety of physiological conditions.

Summary and Conclusion

Dopaminergic responses in the PRL release from lactotrophs have been studied using the rat anterior pituitary cell culture. The PIF activity of hypothalamic extracts was examined in relation to DA.

DA inhibited PRL secretion from the rat anterior pituitary cell culture in a dose-dependent fashion. Its median effective dose was $2 \times 10^{-7}M$ and the maximal inhibition was shown by $10^{-5}M$ DA. The maximal inhibition obtainable by DA was 70-90 % of the control value. The further increase in DA concentration did not result in any further inhibition of PRL secretion.

DA antagonists such as haloperidol and (+)butaclamol could inhibit the effect of DA on PRL release. However, at high concentrations ($10^{-5}M$ or higher), both haloperidol and (+)butaclamol inhibited PRL release. This inhibitory response, however, does not seem to be mediated through dopaminergic mechanism.

High concentrations of both haloperidol and (+)butaclamol (both above $10^{-5}M$) showed some irreversible cytotoxicity on anterior pituitary cells. The major damage seems to be on the plasma membrane. Observations were made at several different levels such as naked eye examination, light microscopy, scanning and transmission electron microscopy.

Hypothalamic extracts also showed PIF activity in the rat anterior pituitary cell culture in a dose-dependent fashion. The maximal concentration of cortical extracts which did not affect the release of PRL from the rat anterior pituitary cell culture was 0.5 hypothalamic weight equivalents. Hypothalamic extracts of 0.5 hypothalamic equivalents showed about 40 % inhibition of PRL release. The effect of the hypothalamic extracts was completely blocked by either 2×10^{-7} M haloperidol or 10^{-6} M (+)butac-
lamol which alone could reverse all the inhibitory effect of 10^{-6} M DA in the same system.

Addition of hypothalamic extracts to DA did not enhance the inhibitory effect of DA, but rather reduced the effect of DA. However, the total inhibitory effect of the combination of hypothalamic extracts and DA was completely reversed by 2×10^{-7} M haloperidol.

It is concluded that all the PIF activity of hypothalamic extracts can be accounted for by dopaminergic activity but not necessarily DA alone. It is possible that other catecholamines such as norepinephrine and epinephrine also exert the PIF activity by acting on the DA receptors in the rat anterior pituitary lactotrophs.

Lastly, the possibility that DA may be a physiological PIF was discussed.

Chapter II

Dopamine in the Rat Anterior Pituitary Organ Culture

Introduction

1. Effect of DA on PRL synthesis

During the last six years, the area of PRL research has been very active, mainly thanks to the availability of PRL RIA kits. Most of the research concerned the effects of various drugs on the release of PRL and their results were reported based on the measurements of immunoreactive PRL in plasma and culture medium.

Since a series of steps are known to be involved intracellularly before a complete PRL molecule appears in the extracellular space, the drug-induced reduction of the PRL level in the extracellular space does not necessarily mean that the drug interferes with the PRL release mechanism.

DA was shown to inhibit PRL release from the rat anterior pituitary organ culture in a dose-dependent fashion (Shaar and Clemens, 1974). Binding studies showed that the rat anterior pituitary has specific DA receptors and the close correlation exists between the binding affinities and abilities to modulate PRL release among dopaminergic agents (Caron et al., 1978). Thus, it seems quite clear that in the rat anterior pituitary DA binds to specific DA sites associated with lactotrophs and that these DA binding sites play a part in modulating PRL secretion.

However, no studies have clearly shown whether the inhibitory effect of DA on PRL secretion is due to its inhibition of the release, or synthesis, or some other mechanisms. The objective of the experiments described in this section is to determine whether DA inhibits PRL synthesis as well as release in the rat anterior pituitary organ culture. [^3H]-Leucine was incubated in the presence or absence of DA, and the newly synthesized PRL was assayed using polyacrylamide disc gel electrophoresis technique.

2. Effect of DA on PRL release pattern

It was shown that there is neither intracellular (Daries et al., 1973) nor extracellular degradation (Tachjian et al., 1970) of PRL during a short-term incubation of the rat anterior pituitary cells. DA was shown

not to interfere with PRL synthesis as will be seen later in this Chapter. Thus, the marked decrease in the medium PRL levels by DA during the anterior pituitary culture seems to be due to its inhibition of PRL release mechanism.

While the effect of DA on PRL synthesis was studied, a very interesting fact was noticed. During the incubation in the presence of [^3H]-leucine, DA inhibited the release of immunoactive PRL by 57 %, while it inhibited the release of newly synthesized PRL by 91 %. This finding seemed to suggest that DA inhibits the newly synthesized PRL preferentially to the pre-existing PRL.

The objective of the experiments described in the present section is to determine whether DA has, indeed, a preferential influence on newly synthesized PRL. This problem has been approached through studying the effects of DA on PRL release patterns during and following the incubation with [^3H]-leucine, in the presence or absence of a protein synthesis inhibitor.

Materials and Methods

Animals

Mature male and female Sprague-Dawley rats (Harlan Industries, Cumberland, Indiana) weighing 250-300 g were used as pituitary donors for all incubations. Animals were maintained in a light-controlled room (lights on from 0600 to 1800 hr), and allowed water and Purina Chow ad libitum.

Experimental procedures

Animals were killed by decapitation and the anterior pituitary was quickly removed and separated from the posterior pituitary. Each anterior pituitary was quartered, distributed equally into four different groups, and weighed prior to preincubation in a 10 ml Erlenmeyer flask. It was previously observed that preincubation could reduce the variations of PRL release among control groups. Each preincubation flask contains four anterior pituitary quarters (each from different animals) and 1.5 ml of medium 199 (Grand Island Biological Co., Grand Island, N.Y.). All the incubations were carried out in a Dubnoff metabolic shaker (60 cpm) at $37.0 \pm 0.5^\circ\text{C}$, under constant gassing with humidified 95 % O_2 - 5 % CO_2 at a rate of 300 cc/min. After 2 hr preincubation, the medium in each flask was removed, the glands rinsed twice with medium 199, and

replenished with 1.5 ml of fresh medium containing a drug or its solvent. The incubation continued for 5-7 hr in the presence or absence of 10 μ Ci L-[4,5-³H]leucine (52 Ci/mmol)(Amersham/Searle). At the end of treatment period, the medium was collected and lyophilized. The glands were homogenized with a glass Ten Broeck homogenizer in 0.05 M KH_2PO_4 (pH = 7.6) solution and lyophilized. The dried samples were stored at -20° C until assayed.

Polyacrylamide disc gel electrophoresis (PADGE)

The dried gland and medium samples were dissolved in 2 ml and 1 ml of sample gel solution, respectively. Aliquots of the gland and medium solutions were subjected to PADGE as described in Davis (1964). Tubes of 75 mm x 5 mm were used. The separating gel was 7.5 % acrylamide, pH 8.8-9.0, and electrode compartments contained Tris-glycine buffer, pH 8.3. Duplicate gels were run for each sample using a current of 3 mA/column. The protein bands were stained in 7 % acetic acid containing 0.5 % Amido Black 10B and excess stain was removed electrophoretically. The location of PRL on the gels was documented by subjecting the reference preparation (NIAMDD rat PRL-RP-1) to the same electrophoretic separation.

Assay

The stained PRL bands were cut from the gel columns and placed in scintillation vials containing 1 ml NCS (tissue solubilizing agent: Amersham/Searle) - water solution (92 : 8, v/v). The capped vials were incubated 4 hr at 50° C. A toluene-based scintillation fluid was added to each vial at room temperature and the samples were counted in a liquid scintillation spectrometer.

The PRL content of incubated glands and media was measured by either densitometry of stained PRL bands, or a RIA using materials and protocols supplied by the NIAMDD Rat Pituitary Hormone Distribution Program (See Appendix B). Each stained gel column was scanned longitudinally in a densitometer (Gilford spectrometer Model 250 equipped with a Model 2410 linear transport carrier) to give a PRL peak and the area under the PRL peak was integrated using a polar planimeter. The results of densitometry were expressed in terms of area under PRL peaks, while the results of RIA were expressed in terms of NIAMD rat PRL-RP-1 (reference preparation).

Drugs

Dopamine HCl (Sigma) was prepared in 0.9 % saline containing 0.05 % sodium metabisulfite and given in a volume of 10 µl. Puromycin 2HCl (Sigma) was prepared in medium 199 and administered in a volume of 50 µl.

Statistics

All data were expressed as means $\pm$ SEM and comparison was made for significant difference between groups using (a) variance homogeneity tests and (b) Student's unpaired t-test for samples of equal or unequal variance.

Results and Discussion

PADGE

Three major bands appear at the end of PADGE of anterior pituitary homogenate as can be seen in Fig. 5. PRL and albumin bands were identified using purified NIH rat PRL and albumin, respectively. A prominent band closest to the origin of sample migration is growth hormone based on the report of Lewis et al. (1969).

1. Effect of DA on PRL synthesis

Effects of DA and puromycin on the PRL levels in the rat anterior pituitary culture

Following 10^{-5} M DA treatment, the amount of PRL released into the medium decreased significantly ($p < 0.01$), while the amount of PRL in the gland increased significantly ($p < 0.01$) as compared to control group (Table 11). However, the total amount of PRL, i.e., gland and medium, was not significantly ($p > 0.05$) different from control group (Table 11). These results seem to suggest that DA does not inhibit PRL synthesis, but inhibits the release of PRL, which results in accumulation of PRL in the gland.

On the other hand, 10^{-4} M puromycin treatment resulted in a significant resuction ($p < 0.01$) in both medium and gland PRL levels. Since the total amount of PRL in the system was significantly ($p < 0.01$) reduced, the decrease in

Figure 5. The electrophoretic pattern of the rat anterior pituitary gland homogenate. The homogenate (left) of 0.4 mg equivalent of an anterior pituitary gland and 10 μ g of the reference preparation (right), NIAMD rat prolactin-RP-1, were subjected to polyacrylamide disc gel electrophoresis. GH: growth hormone, ALB: albumin, PRL: prolactin

TABLE 11

Effect of DA and Puromycin on the PRL Levels
in the Rat Anterior Pituitary Culture

Treatments	PRL (cm ² /mg pituitary) ^a		
	Medium	Gland	Medium+Gland
Control	5.5±0.3	19.0±0.3	24.5±0.6
DA(10 ⁻⁵ M)	1.8±0.3	25.4±1.5	27.2±1.8
Puromycin(10 ⁻⁴ M)	3.3±0.1	9.8±0.1	13.1±0.2

^a Each value represents a mean ± SEM of two incubation flasks and is expressed as the area under the PRL peak during densitometry of PRL band. Each flask contained an equivalent of one female rat anterior pituitary and was incubated 4.5 hr.

medium PRL level seems to be due to the inhibition of PRL synthesis.

Effects of DA on PRL synthesis and release in the rat anterior pituitary culture

In this experiment, [^3H]-leucine was added during incubation, in order to study the synthesis of [^3H]-PRL (hence, newly synthesized PRL) in the presence and absence of DA. As can be seen in Fig. 6, significant amount of [^3H]-leucine was incorporated into PRL molecules during the incubation. DA decreased the amounts of the total and newly synthesized PRL in the medium by 57 % and 91 %, respectively, while it increased the amounts of the total and newly synthesized PRL in the gland by 152 % and 188 %, respectively, as compared to control group. However, the total amount of PRL, newly synthesized or total, was not significantly ($p > 0.05$) different between control and DA group. Since it was previously shown that PRL molecules are not degraded either intracellularly (Daries et al., 1973) or extracellularly (Tashjian et al., 1970), it seems clear that (a) DA does not influence the PRL synthesis, (b) but only suppresses the release of PRL, which results in accumulation of PRL in the gland.

The present results are inconsistent with previous results of MacLeod and Lehmeyer (1974a). These authors showed that both the total and released amount of newly

Figure 6. Effect of DA on PRL synthesis and release in the rat anterior pituitary organ culture. Pituitaries from male rats were preincubated for 2 hr and the incubation continued for another 7 hr in medium 199 containing [4,5-³H]leucine in the presence or absence of 10⁻⁵M DA. The amount of newly synthesized PRL was expressed in terms of disintegration per minutes (dpm) of electrophoretically separated PRL bands, while the amount of the total PRL was expressed in terms of NIAMD rat PRL-RP-1 (reference preparation). The open and dotted bars represent means of gland and medium PRL levels from duplicate flasks, respectively. One SEM was drawn for each bar except those with very small SEM's.

synthesized PRL were significantly decreased by DA, suggesting that DA inhibits synthesis as well as release of PRL. This major discrepancy seems to be due to the presence and absence of preincubation. MacLeod and Lehmeyer (1974a) incubated 6-7 hr without any preincubation, while the present experiments had 2 hr preincubation prior to 5-7 hr treatment period.

It is well known that the PRL secretion from lactotrophs is under tonic inhibitory influences in vivo and the removal of this inhibition can result in marked secretion of PRL. The preincubation phase may have depleted some of the intracellular PRL pool. As can be seen in Table 11 and Fig. 6, the fact that DA can increase both total and newly synthesized PRL contents in the gland as compared to control, seems to indicate that, normally, the lactotroph PRL pools are almost fully occupied with PRL molecules due to the consistent presence of DA in the hypophyseal portal blood. Thus, the results of MacLeod and Lehmeyer (1974a) seem to indicate that the high intracellular PRL contents due to lack of preincubation period caused the inhibition of the further synthesis of PRL.

The following evidence seem to support the view that the intracellular PRL levels control its further synthesis.

First, incubation of female rat anterior pituitary glands for 7 hr in the presence of norepinephrine without any preincubation, resulted in a significant reduction of

the amount of newly synthesized PRL as compared with control (MacLeod, 1969). However, measurements of PRL during the first 2 hr of norepinephrine treatments showed no reduction in the total amount of newly synthesized PRL despite the decrease in medium and increase in gland newly synthesized PRL levels (MacLeod, 1969). Since it was previously shown that norepinephrine inhibits PRL release by acting on lactotroph DA receptors (See Chapter I), these results seem to indicate that the lactotrophs continue to synthesize PRL while DA inhibits the release of PRL, until the intracellular PRL pools are saturated.

Secondly, after pituitary stalk section in female rats, the plasma PRL level rose greatly (Kanematsu et al., 1979). The electron microscopy of lactotrophs from these rats showed not only a marked decrease in PRL granules, but also a great increase in immature PRL granules in the well developed Golgi area (Kanematsu et al., 1979). Since the anterior pituitary is normally under the control of DA, the suppression of PRL synthesis under the normal situation seems to be due to inhibition of PRL release by DA.

In light of the aforementioned facts, the inhibition of PRL synthesis by DA as was shown in MacLeod and Lehmeyer (1974) seems to be due to a high intracellular PRL level, which acts to inhibit the further synthesis of PRL.

2. Effect of DA on PRL release pattern

A ratio of $\frac{*PRL}{PRL}$ as a tool to study the release pattern of PRL

The principle of this method is based on the assumption that, once leucine and [3H]-leucine are mixed in a fixed ratio at the beginning of the incubation, the same ratio will be applied in every single step involved in the PRL synthesis such as uptake, transport, binding to t-RNA, and finally, the formation of PRL molecules.

If lactotrophs start synthesizing PRL molecules in the presence of [3H]-leucine in addition to other ingredients of the regular culture medium, the relationship between newly synthesized PRL (P_n) and pre-existing PRL (P_o) can be derived as follows:

- *L : number of molecules of [3H]-leucine
- L : a total number of molecules of leucine, both labeled and unlabeled
- *P : radioactivity in dpm of the newly synthesized PRL
- P_n : number of molecules on newly synthesized PRL
- P_o : number of PRL molecules existing prior to incubation in the presence of [3H]-leucine
- P : number of PRL molecules, both labeled and unlabeled
- k_a : number of leucine molecules in one PRL molecule
- k_c : radioactivity in dpm of one [3H]-leucine molecule

$$\frac{{}^*L \text{ in medium}}{L \text{ in medium}} = \frac{{}^*L \text{ in Pn}}{L \text{ in Pn}} = \frac{{}^*L \text{ in Pn}}{k_a \times Pn} = k_b$$

$${}^*L \text{ in Pn} = k_a \times k_b \times Pn$$

On the other hand,

$$\begin{aligned} {}^*P &= ({}^*L \text{ in Pn}) \times k_c \\ &= (k_a \times k_b \times Pn) \times k_c \\ &= K \times Pn \end{aligned} \quad \text{--- (1)}$$

Thus, the radioactivity of PRL is directly proportional to the amount of newly synthesized PRL.

Anytime during incubation period,

$$P = Pn + Po \quad \text{--- (2)}$$

by dividing eq.(1) by (2),

$$\frac{{}^*P}{P} = K - \frac{K Po}{P} \quad \text{--- (3)}$$

Eq.(3) can be expressed graphically in Fig. 7.

Since P increases with the period of incubation (t), substitution of time for P will result in a similar graph. It can be seen from the figure, the ratio of ${}^*P/P$ increases as the incubation time increases, finally approaching a constant ratio, K.

In reality, the system has two major PRL compartments, the intracellular and extracellular ones. During the

Figure 7. Relationship between the ratio of newly synthesized prolactin to total prolactin ($*P/P$) and total prolactin (P) or incubation time (t).

incubation period, PRL will be continuously synthesized and released along with the pre-existing PRL. Theoretically, as the time goes on, more and more synthesized PRL will be released than the pre-existing one, eventually a ratio of $^*P/P$ in either compartments approaching a constant, K .

Suppose a drug inhibits only the release of PRL throughout the incubation period. The comparison of the ratios of $^*P/P$ in the presence or absence of the drug may be able to show whether the drug have any preference for the release of one type of PRL to the other, i.e., newly synthesized or pre-existing PRL. The method will be discussed later by using a hypothetical model for PRL dynamics.

A model for PRL dynamics

Almost no studies have been reported regarding PRL dynamics except the studies of MacLeod and Abad (1968). They reported the time course on synthesis and release of newly synthesized PRL as can be seen in Fig. 8. An attempt has been made in the present experiment to establish a model for PRL dynamics based on Fig. 8, and the results of the present experiments regarding effects of DA on PRL release pattern have been examined.

Several characteristics can be found from Fig. 8.

1. The total amount of *P is almost linearly increasing

Figure 8. Distribution of newly synthesized PRL between the pituitary gland and incubation medium. Reproduced from MacLeod and Abad (1968).

vs. time, indicating that the rate of synthesis is linear with time.

2. The contents of *P in gland show hyperbolic function with time while the rate of *P release into the medium was slow at the beginning but linear after the gland *P contents become almost constant. This indicates that the *P release may be a first order function of *P contents of the gland.

Although other mathematical functions could be possible in explaining Fig. 8, the hypothesized functions were tested first to see whether they can resemble Fig. 8. Suppose *P is synthesized in the gland at a zero order rate constant k_1 , and both P_n and P_o in the gland are released into the medium at first order rate constants k_2 and k_3 , respectively.

$$\frac{d(P_n)}{dt}_g = k_1 - k_2(P_n)_g \quad \text{--- (4)}$$

where g represents gland,

$$\frac{d P_o(t)}{dt}_g = - k_3 P_o(t)_g \quad \text{--- (5)}$$

Solving the differential eq.(4),

$$(P_n)_g = k_1/k_2 - C e^{-k_2 t}$$

(C is a constant of integration)

Since at $t=0$, $P_n=0$,

$$C = k_1/k_2$$

$$(P_n)_g = k_1/k_2 (1 - e^{-k_2 t}) \quad \text{--- (6)}$$

Substituting eq.(1) into eq.(6),

$$(^*P)_g = K k_1/k_2 (1 - e^{-k_2 t}) \quad \text{--- (7)}$$

Solving eq.(5),

$$P_o(t)_g = P_o e^{-k_3 t} \quad \text{--- (8)}$$

Since $P = P_n + P_o(t)$,

$$(P)_g = k_1/k_2 (1 - e^{-k_2 t}) + P_o e^{-k_3 t}$$

Thus,

$$(^*P/P)_g = \frac{K k_1/k_2 (1 - e^{-k_2 t})}{k_1/k_2 (1 - e^{-k_2 t}) + P_o e^{-k_3 t}} \quad \text{--- (9)}$$

On the other hand, in the medium,

$$(^*P)_m/dt = k_2 (^*P)_g \quad \text{--- (10)}$$

$$(P)_m/dt = k_2 (P_n)_g + k_3 P_o(t)_g \quad \text{--- (11)}$$

Where m represents medium.

Solving eq.(10) by substituting eq.(7),

$$\begin{aligned}
 (^*P)_m &= k_2 \int K k_1/k_2 (1-e^{-k_2 t}) dt \\
 &= K (k_1 t + k_1/k_2 e^{-k_2 t}) + C
 \end{aligned}$$

(C is a constant of integration)

Since at $t=0$, $(^*P)_m = 0$,

$$C = - K k_1/k_2$$

$$(^*P)_m = K k_1 t - k_1/k_2 (1-e^{-k_2 t}) \quad - - - - - (12)$$

Solving eq.(11) by substituting eq.(6) and (8),

$$\begin{aligned}
 (P)_m &= k_2 (Pn)_g dt + k_3 Po(t)_g dt \\
 &= k_1 t - k_1/k_2 (1-e^{-k_2 t}) + Po(1-e^{-k_3 t})
 \end{aligned}$$

Therefore,

$$(^*P/P)_m = \frac{K k_1 t - k_1/k_2 (1-e^{-k_2 t})}{k_1 t - k_1/k_2 (1-e^{-k_2 t}) + Po(1-e^{-k_3 t})} \quad - - (13)$$

In order to see whether the present model fits the experimental data (Fig. 8), eq.(7) and (12) were plotted against time (Fig. 9). It can be seen that Fig. 9 (gland and medium curves) resembles Fig. 8 very closely. Thus, the present model may be very usefully employed in examining some significant changes in experimental data due to drug treatments such as DA.

Figure 9. Distribution of newly synthesized PRL between the pituitary gland and incubation medium based on a hypothetical model for PRL dynamics.

Effect of DA on PRL release pattern

Consistent with the assumption of independence of k_3 from k_1 and k_2 , eq.(7) and (12) show that the amounts of newly synthesized PRL in both the gland and the medium are not influenced by a rate constant for the release of the pre-existing PRL, k_3 , as long as k_3 does not alter the kinetics of the newly synthesized PRL. In fact, k_3 can be any order kinetics without changing Fig. 9.

Suppose the pre-existing PRL is released in a first order kinetics, as was assumed in eq.(5). If k_3 is equal to k_2 , that is to say, $(^*P)_g$ and $(Po)_g$ are either mixed in the same pool and released at a first order rate constant, k_2 , or released from separate pools at a same first order constant, k_2 , then after incubation for time, t ,

$$(^*P/P)_g = \frac{K k_1/k_2(1-e^{-k_2t})}{k_1/k_2(1-e^{-k_2t}) + Po e^{-k_2t}} \quad - - - - - (14)$$

$$(^*P/P)_m = \frac{K k_1t - k_1/k_2(1-e^{-k_2t})}{k_1t - k_1/k_2(1-e^{-k_2t}) + Po(1-e^{-k_2t})} \quad - - (15)$$

Two characteristics can be found from these equations.

1. The ratio of $^*P/P$ approaches a constant, K , after infinitesimal time period.

2. $(^*P/P)_g > (^*P/P)_m$ at any time during incubation.

See Appendix C for calculation.

Table 12 shows that the ratio of $^*P/P$ of the control group after 7 hr incubation in the presence of [3H]-leucine is higher in the medium than gland. The same was true in the control group of Table 14 in the presence of puromycin. The case of puromycin treatment will be discussed later in this section. Thus, the original assumption that k_2 is equal to k_3 should be wrong.

The alternative assumption could be that k_2 is greater than k_3 , that is, the rate constant for the release of newly synthesized PRL is greater than that for the pre-existing PRL, implying the existence of two different release mechanisms. It would be more reasonable to assume two different pools for PRL release rather than one single pool, since it is very unlikely that the labeled PRL is selectively released from a pool containing both labeled and unlabeled PRL. Under the situation that k_2 is greater than k_3 , $(^*P/P)_m$ would be much smaller than $(^*P/P)_g$ initially, since a certain time period is required before *P becomes available for release into the medium. However, $(^*P/P)_m$ would increase gradually during incubation, eventually becoming greater than $(^*P/P)_g$. This seems to be the case in the present in vitro pituitary organ culture, judging from the values of control groups in Tables 12, 13, and 14.

The presence of DA either during or following the incubation with [3H]-leucine markedly reduced the value of

TABLE 12
Effect of DA on PRL Release Pattern
During Incubation with [³H]-leucine

Groups		*PRL(dpm)/PRL(ng) ^a
Control	Medium	1.167 ± 0.020
	Gland	0.760 ± 0.022
DA (10 ⁻⁵ M)	Medium	0.248 ± 0.054
	Gland	0.835 ± 0.001

^a Values represent means ± SEM calculated from Fig. 6. For details, see Fig. 6.

TABLE 13
Effect of DA on PRL Release Pattern
Following Incubation with [^3H]-leucine

Groups		*PRL(dpm)/PRL(ng)
5 hr	Medium (2)	0.310 $\pm$ 0.021
	Gland (2)	0.435 $\pm$ 0.040
Control	Medium (3)	0.707 $\pm$ 0.066
	Gland (3)	0.360 $\pm$ 0.015
DA (10^{-5}M)	Medium (3)	0.241 $\pm$ 0.051
	Gland (3)	0.422 $\pm$ 0.040

Pituitaries from male rats were preincubated for 2 hr and the incubation continued in medium 199 containing [^3H]-leucine for 5 hr. At the end of the incubation with the labeled leucine, the 5 hr samples were taken. All the other flasks were rinsed three times with medium 199 and reincubated in [^3H]-leucine free medium 199 for 5 hr in the presence or absence of DA. Values represent means $\pm$ SEM, and the numbers in parentheses represent the number of incubation flasks.

TABLE 14

Effect of DA on PRL Release Pattern in the Presence of Puromycin Following Incubation with [3H]-leucine

Groups		*PRL(dpm)/PRL(ng)
Control	Medium	1.703 ± 0.167
	Gland	0.850 ± 0.019
DA (10 ⁻⁵ M)	Medium	0.769 ± 0.007
	Gland	1.099 ± 0.046

Pituitaries from male rats were preincubated for 2 hr and the incubation continued for 5 hr in medium 199 containing [4,5-³H]leucine. Thirty minutes before the end of the 5.5 hr incubation, 10⁻⁴M puromycin was added to all the flasks. It was previously shown that 10⁻⁴M puromycin could completely inhibit PRL synthesis in 30 minutes. At the end of the incubation with the labeled leucine, all the flasks were rinsed with medium 199 and reincubated in [4,5-³H]leucine free medium 199 for 5 hr in the presence or absence of DA. Values represent means ± SEM of three incubation flasks.

*P/P in the medium, while increased that in the gland as compared to control (Tables 12 and 13). Thus, DA seems to inhibit PRL release with a preference on newly synthesized PRL to the pre-existing PRL. This interpretation becomes conclusive in the puromycin study (Table 14).

During the chase period following incubation with [³H]-leucine, a significant amount of newly synthesized PRL was released and the release was greatly blocked by DA (Table 13). The continuous release of the newly synthesized PRL during the chase period could be due partly to the continued synthesis of PRL from the intracellular leucine pool. Thus, complete blockade of the translation step in PRL synthesis would solve the problem of the continued synthesis, and make it possible to see the release pattern of PRL more accurately.

It has been shown that puromycin ($10^{-4}M$) could completely inhibit PRL synthesis. Assuming that puromycin does not interfere with PRL release mechanism, then in the presence of puromycin following incubation with [³H]-leucine

$$d(*P)_g/dt = -k_2(*P)_g \quad \text{--- (16)}$$

$$d(P)_g/dt = -k_2(Pn)_g - k_3 P_0(t)_g \quad \text{--- (17)}$$

Solving eq.(16),

$$(*P)_g = (*P')_g e^{-k_2 t} \quad \text{--- (18)}$$

where, $^*P'$ is the radioactivity of Pn immediately before PRL synthesis is blocked by puromycin.

Solving eq.(17), and at the same time substituting $(Pn)_g = (Pn')_g e^{-k_2 t}$ and $Po(t)_g = (Po')_g e^{-k_2 t}$ which are derived from the same equations as eq.(16) in case of Pn and Po,

$$(P)_g = (Pn')_g e^{-k_2 t} + (Po')_g e^{-k_3 t} - - - - - (19)$$

where, Pn' and Po' represent the numbers of molecules of newly synthesized and pre-existing PRL, respectively, before PRL synthesis is blocked by puromycin.

Since $^*P = K Pn$ from eq.(1),

$$(^*P/P)_g = \frac{K(Pn')_g e^{-k_2 t}}{(Pn')_g e^{-k_2 t} + (Po')_g e^{-k_3 t}} - - - - - (20)$$

On the other hand, in the medium,

$$d(^*P)_m/dt = k_2(^*P)_g - - - - - (21)$$

$$d(P)_m/dt = k_2(Pn)_g + k_3 Po(t)_g - - - - - (22)$$

Solving eq.(21) and (22),

$$(^*P/P)_m = \frac{K(Pn')_g (1-e^{-k_2 t})}{(Pn')_g (1-e^{-k_2 t}) + (Po')_g (1-e^{-k_3 t})} - - - (23)$$

If k_2 is equal to k_3 , that is, newly synthesized and pre-existing PRL are released with the same rate constant, the ratios of $^*P/P$ in eq.(20) and (23) become equal and

constant for both gland and medium as can be seen in eq.(24).

$$(*P/P)_g = (*P/P)_m = \frac{K (Pn')}{(Pn') + (Po')} \quad - - - - - (24)$$

However, the control group of Table 14 shows that $(*P/P)_m$ is remarkably greater than $(*P/P)_g$ and vice versa in the gland. Thus, K_2 should be greater than k_3 .

The values of $*P/P$ were even reversed in the presence of DA, i.e., $(*P/P)_g$ being greater than $(*P/P)_m$. This marked reduction of $(*P/P)_m$ in the presence of DA seems to clearly indicate that DA inhibits the release of newly synthesized PRL in preference to the pre-existing PRL.

The following model may be hypothesized regarding the synthesis and release of PRL in the lactotrophs.

1. Normally, PRL seems to be synthesized at a zero order rate.
2. Most of the newly synthesized PRL may be transferred, inside secretory granules, into one type of the secretory pool. The secretion of PRL from this pool seems to be very active and suppressed by DA.
3. Most of the pre-existing PRL and probably a small portion of newly synthesized PRL may be stored in a separate pool. The secretion of PRL from this pool seems to be less active and not inhibited by DA.
4. High intracellular levels of newly synthesized PRL may suppress its further synthesis.

Summary and Conclusion

Effects of DA on PRL synthesis as well as release were studied in the rat anterior pituitary organ culture.

DA suppressed the release but increased the anterior pituitary gland contents of both radioimmunoassayable and newly synthesized PRL. The total PRL synthesis was not affected by DA. These results indicate that DA does not directly inhibit PRL synthesis, and the increase in PRL contents of the anterior pituitary gland is due to the inhibition of PRL release by DA.

The release pattern of PRL was studied in the rat anterior pituitary organ culture, using a ratio of newly synthesized to total PRL contents in the gland and the medium. A model for PRL dynamics was developed based on experimental data. There seem to be two different types of PRL secretory pools in the lactotrophs. The newly synthesized PRL seems to be contained in one type of pools and very actively secreted, while the pre-existing PRL seems to be contained in the other type of pools and less actively secreted. DA seems to preferentially inhibit the release of the newly synthesized PRL.

Chapter III

Prolactin "Surge" in the Rat Anterior Pituitary Monolayer Culture Following Medium Replacement

Introduction

Like other anterior pituitary hormones, PRL seems to be capable of regulating its own secretion. It has been reported that transplantation of normal pituitary glands to the kidney capsule (Mena et al., 1968; Sinha and Tucker, 1968), implantation of PRL producing tumors into female rats (MacLeod et al., 1966; Chen et al., 1967), or daily injection for 10 days with ovine PRL (Sinha and Tucker, 1968) leads to a reduction in pituitary content of PRL in the primary pituitary of the recipient animals and reduce the ability of the pituitary to synthesize (MacLeod and Abad, 1968; MacLeod and Lehmyer, 1974b) and release new PRL (MacLeod and Lehmyer, 1974b).

Similar observations were made in rats after the implantation of PRL into the median eminence (Clemens and

Meites, 1968; Welsch et al., 1968). PRL was found in the cerebrospinal fluid both in rats treated with haloperidol (Login and MacLeod, 1977) and in humans (Assies et al., 1978), and increased PRL in the cerebrospinal fluid increased DA turnover in the median eminence (Annunziato and Moore, 1978), suggesting that the mechanisms regulating the short loop feedback system of PRL control might be mediated via the hypothalamus.

Recently, Herbert et al. (1979) reported that PRL could inhibit its own secretion through direct action on the lactotrophs, using his monolayer culture of a pure clonal strain of fetal pituitary cells. However, opposite findings were reported in the anterior pituitary organ culture (Voogt and Ganong, 1974), in the cell culture (Groshong et al., 1977), and in the isolated anterior pituitary "perifusion" system (Maltz et al., 1978). Thus, the view that the pituitary serves also as a site for the autoregulation of PRL is somewhat uncertain.

It has been reported by Groshong et al. (1977) and Deneff and Follebouck (1978) that, during the anterior pituitary cell culture, changing the culture medium caused a marked PRL surge during the first hour. Since the autoregulation of PRL at the pituitary level is uncertain, it would be interesting to see whether the PRL surge following medium replacement is caused by the removal of the possible negative feedback by PRL.

The objective of the present experiments is to determine what causes the PRL surge following medium replacement in the rat anterior pituitary monolayer culture. In solving this question, three possible causes have been examined:

1. removal of a high concentration of PRL,
2. removal of metabolites, which may have been suppressing PRL release,
3. mechanical stress involved in changing the medium.

Materials and Methods

Animals and cell dispersion techniques have been previously described in Chapter I.

Experimental protocol

An eleven day old culture was divided into six groups each containing three culture wells. All the wells containing 1 ml medium, were preincubated for 6 hr. At the end of the preincubation period, a 0.1 ml aliquot of the cultured medium was withdrawn from each well for the purpose of RIA. The remaining medium (0.9 ml) in each well of groups 3-6 was removed by suction using sterile pipets. Groups were treated as follows:

- Group 1. no medium change
2. medium was withdrawn and the same medium was added back.
3. 0.9 ml fresh medium was added.
4. 0.9 ml of preincubated medium was added.
5. 0.8 ml of fresh medium + 0.1 ml 1% BSA buffer^a.
6. 0.8 ml of fresh medium + 100 ng NIAMDD rat PRL-RP in 1% BSA buffer.
7. 0.9 ml preincubated medium in cell-free wells in order to monitor the possible volume loss during incubation.

^a See Appendix B.

All the media added were prepared just prior to the preincubation and stored inside the same incubator as the culture wells were, in an attempt to reduce the possible effects of temperature and pH of the different media during treatment (about 30 min outside the incubator). Incubation continued for 12 hr, and a 0.1 ml aliquot of medium was sampled from each well, at 1, 2, 6, and 12 hr following incubation. The samples were immediately frozen at -20°C until assayed for PRL content using RIA described in Chapter I.

Results and Discussion

It was previously shown by our laboratory that PRL contained in the culture medium is not degraded when incubated in a cell-free container up to 12 hr. As can be seen in group 7 of Table 15, the amount of PRL contained in a 0.1 ml aliquot of the preincubated medium was not significantly changed during the 6 hr incubation period, indicating that there is no significant change in incubation volume due mainly to evaporation during incubation. Knowing the exact volume is very important in a study like the present one in which a total amount of PRL in each well is calculated from a fraction of each well volume.

The present experiment did not show any PRL surge during the first hour following medium replacement, as can be seen in group 3. After changing the medium with a fresh one, PRL was released at about the basal rate judging from the values of 2, 6, and 12 hr in group 3.

Groshong et al. (1977) reported that,

"Changing the culture media after 24-72 hr of incubation resulted in a 22 fold increase in the PRL secretion rate during the first hour."

In interpreting their data, at least three facts should have been considered.

1. PRL molecules are not immunologically stable after 24 hr incubation. It was previously observed in our laboratory that a 24 hr incubation of a known amount of

TABLE 15
PRL Release Following Medium Change

Treatments	% PRL release/PI ^a			
	1 hr	2 hr	6 hr	12 hr
1. No M.C. ^b	0.5 ± 0.1	16.6 ± 0.6	58.9 ± 3.4	172.1 ± 7.8
2. Psedo M.C. ^c	12.4 ± 3.6	31.3 ± 3.8	74.2 ± 2.4	194.5 ± 12.0
3. F.M.C. ^d	17.5 ± 2.5	30.3 ± 2.4	140.1 ± 1.0	206.2 ± 5.8
4. P.M.C. ^e	43.8 ± 6.8	52.3 ± 5.6	67.8 ± 0.9	127.7 ± 11.8
5. F.M.C. + 1% BSA ^f	9.2 ± 0.8	16.7 ± 0.7	79.6 ± 5.1	101.0 ± 6.6
6. F.M.C. + 100ng PRL ^g	10.1 ± 1.8	13.8 ± 4.2	84.3 ± 5.2	100.5 ± 2.2
7. P.M. ^h	2.88±0.12	2.78±0.12	2.75±0.10	

Eleven day old monolayer culture from male rats were used. Cells were preincubated (PI) for 6 hr, treated as indicated, and reincubated in 0.9 ml medium for 12 hr. During the incubation period, the medium was withdrawn at 1, 2, 6, and 12 hr from each culture well.

^a The amount of PRL released during treatment period was divided by the amount of PRL released during the PI period. Each value represents a mean ± SEM of three culture wells.

TABLE 15 (continued)

- ^b Medium was not changed.
- ^c removed the medium and added back the same medium.
- ^d changed the medium with fresh one.
- ^e changed the medium with the preincubated one which contained 350 ng PRL/ml medium.
- ^f A 0.1 ml aliquot of 1% bovine serum albumin phosphate buffer (See Appendix B) was added to 0.9 ml of fresh medium.
- ^g A 0.1 ml aliquot of NIAMDD rat PRL-RP-1 100 ng dissolved in 1% BSA buffer was added to 0.9 ml of fresh medium.
- ^h The preincubated medium containing 350 ng PRL/ml was added into cell-free wells and incubated under the same incubation conditions as other treatment groups were. A 0.1 ml aliquot of the medium was sampled at 1, 2, and 6 hr following incubation. The values represent means $\pm$ SEM (n=3) of the amounts of PRL contained in 0.1 ml aliquots of 12X diluted samples.

PRL resulted in a reduction of its immunoactivity by about 40 %. Thus, a long term incubation such as 24-72 hr will markedly reduce the basal secretion rate, since the rate was calculated in terms of the total amount of PRL secreted during the 24-72 hr divided by the number of hours incubated.

2. A single wash performed by Groshong^a may not remove all the PRL in the medium. At the end of the long term incubation, a large amount of PRL will be present in the culture medium. A little leftover following the medium change could be large enough to affect the first hour secretion rate which was shown to be very small in the present experiment.

3. The long term incubation may have suppressed the secretory activity of the lactotrophs because of some unfavorable culture conditions such as accumulation of metabolites, acidic pH, nutrient supply, and possible negative feedback by accumulated PRL. The subsequently accumulated PRL inside the cells may be subjected to a sudden release in a fresh medium.

During the first hour of the incubation, a group without medium change (group 1) showed a significantly lower PRL release than a group with fresh medium change (group 3). This initial suppression of PRL secretion rate could be due to unfavorable incubation conditions, because

^a personal communication

the wells were in the ambient environment during a 30 min treatment period. As a result, temperature of the culture medium must have been lowered and enough CO₂ escaped to raise the pH of the medium. Despite the same unfavorable conditions as group 1 had, a group with fresh medium change showed a higher PRL secretion rate than group 1 during the first hour of incubation.

This increase of PRL secretion rate could be due to (a) removal of negative feedback by the already secreted PRL in the medium, or (b) removal of metabolites from the medium, or (c) the presence of a mechanical stress.

Herbert et al. (1979) showed that ovine PRL ranging from 0.01 to 1000 ng/ml medium could inhibit PRL release in the rat anterior pituitary monolayer culture, in a dose-dependent fashion. However, 100 ng rat PRL did not inhibit the PRL release during the first hour of incubation (group 6). Both pseudo and preincubated medium change groups (groups 2 and 4) which contained large amounts of PRL in the medium did not suppress the initial PRL secretion rate. Thus, the increase in the initial PRL secretion rate in group 3 does not seem to be due to the removal of the negative feedback by the large amount of PRL in the medium.

Both pseudo and preincubated medium change groups which should contain metabolites did not suppress the initial PRL release, which indicates that the initial PRL

Summary and Conclusion

During the rat anterior pituitary monolayer culture, changing the medium did not cause a PRL surge as had been reported by others. However, a group of culture wells which continued to be incubated without changing the medium secreted a remarkably smaller amount of PRL than one with fresh medium replacement, during the first hour of the incubation.

Possible causes of the increase in the initial secretion rate following fresh medium replacement were examined under several different experimental designs. It was found that the initial enhanced secretion was caused neither by the removal of the high PRL concentrations nor metabolites in the medium, but by the simple mechanical stress involved in changing the medium.

APPENDIX A

Preparation of collagenase solution

Collagenase (type A)^a was dissolved in Ca^{++} and Mg^{++} free Hank's base^b such that the concentration became approximately 1 % (w/v). The solution was filtered through 0.45 μm sterile membrane-filter and the filtrate was diluted, if necessary, with sterile double distilled water such that the final concentration became 1 %. Aliquots of 3.5 ml of the final solution were distributed into sterile screw-capped culture tubes and frozen immediately at -20°C , until used.

Preparation of culture medium

Powdered Minimum Essential Medium (MEM)(EAGLE)^c was dissolved in 300 ml of double distilled water and 0.35 g of NaHCO_3 was added. The MEM solution was filtered through 0.2 μm sterile membrane-filter. This MEM solution was stored at 4°C , not more than 7 days before use. Immediately before use, the other ingredients were added in the following concentrations:

release is not due to the removal of the metabolites from the medium.

The insufficient supply of nutrients during the first hour of incubation of group 1 does not seem to be a very likely explanation, since fresh medium had been added only 6 hr before the actual incubation, and the PRL secretory rate is almost linear up to 12 hr incubation as can be seen in group 3.

However, changing the medium itself did increase the initial secretion rate, regardless of the types of medium changed. Thus, the initial PRL release may be due mainly to the mechanical stress involved in physically changing the medium.

0.1 mM non-essential amino acids^d, 1 mM sodium pyruvate^e,
10 % fetal bovine serum^f, and 40 mg gentamicin^g/l medium.

^a Worthington Biochemical Corp.

^b Hank's balanced salt solution
Cat # 310-4180, Grand Island Biological Co. (GIB)

^c MEM(EAGLE) powdered tissue culture medium
Cat # 320-1140, GIB.

^d MEM nonessential amino acids
Cat # 320-1140, GIB.

^e MEM sodium pyruvate solution
Cat # 320-1360, GIB.

^f Fetal calf serum
Cat # 614, GIB.

^g Garamycin, injectable (80 mg/2 ml)
Schering Pharmaceutical Corp.

APPENDIX B

Radioimmunoassay of Rat Prolactin

Reagents provided by NIAMDD

1) Purified rat prolactin for radioiodination:

NIAMD-Rat Prolactin-I-3

Biological potency = approximately 30 International
units/mg. (crop sac assay)

GH contamination = less than 0.1 International
Units/mg. (hypophysectomized
rat body weight gain assay)

TSH contamination = less than 0.1 USP Units/mg.
(McKenzie assay)

100 µg is provided, lyophilized.

2) Antiserum to rat prolactin, prepared in rabbits:

NIAMD-Anti-Rat-Prolactin Serum-6

400 µl, undiluted, is provided in liquid form.

The antiserum is stable in this form. Freeze
immediately after receipt.

3) Rat prolactin reference preparation for radioimmunoassay

NIAMD-Rat Prolactin-RP-1

Biological potency = approximately 11 International
Units/mg. (mouse deciduoma assay)

Approximately 1 mg of lyophilized material is provided.

Storage of reagents

1) Purified rat PRL (NIAMD-Rat PRL-I-3)

Solubilize in 0.05M sodium phosphate buffer at 200 µg/ml. Transfer aliquots of 15 µl into 400 µl polyethylene microtubes with stoppers and snap-freeze the tubes by immersion in a mixture of dry ice and ethanol. Store the frozen aliquots at -20° C.

2) Antiserum to rat PRL (NIAMD-Anti-Rat PRL-S-6)

Prepare a 1 : 10 dilution of the antiserum with 3 % normal rabbit serum buffer. Transfer aliquots of 100 µl to the 400 µl polyethylene microtubes, stopper the tubes, snap-freeze, and store at -20° C.

3) Reference preparation for RIA (NIAMD-Rat PRL-RP-1)

Weigh a small amount of the lyophilized powder.

Dissolve in 1 % bovine serum albumin phosphate (BSA) buffer and dilute such that the final concentrations become 10, 5, 2.5, 1.25, 0.625, 0.3125 ng per 100 µl buffer solution. Transfer aliquots of 350 µl from each concentration into the 400 µl microtubes,

stopper the tubes, snap-freeze, and store at -20°C .

Iodination of rat prolactin

Reagents

0.5 M sodium phosphate buffer, pH 7.6.

Rat PRL(NIAMD-Rat PRL I-3) (2 $\mu\text{g}/10\ \mu\text{l}$ of 0.05 M sodium phosphate buffer).

^{125}I , carrier free, with specific activity of about 100 mCi/ml, suitable for iodination of protein.

If necessary, dilution can be made with 0.05 M sodium phosphate buffer.

Chloramine-T (15 mg/10 ml of 0.05 M sodium phosphate buffer, pH 7.6). The chloramine-T solution is prepared just prior to actual use.

Sodium metabisulfite (25 mg/10 ml of 0.05 M sodium phosphate buffer, pH 7.6). This solution is also prepared just prior to use.

0.05 M sodium barbiturate buffer, pH 8.6.

Labeling procedures

In a 10 x 75 mm siliconated disposable glass culture tube, the reagents are added in a following order.

- 1) 20 μl of 0.5 M phosphate buffer
- 2) 10 μl of the rat PRL solution
- 3) 0.5 mCi of ^{125}I
- 4) 10 μl of chloramine-T solution.

Immediately following addition, the tube is

is agitated 20 to 25 seconds after which,
5) 100 μ l of sodium metabisulfite solution is quickly added and mixed to terminate the iodination.

The entire reaction mixture is applied to a Sephadex G-50 column, prepared as described below. The column is eluted with 0.05 M sodium barbiturate buffer and fractions of 1.5 ml are collected in 18 x 150 mm glass culture tubes, coated with approximately 20 μ l of 30 % (w/v) bovine serum albumin solution.

Results

Two peaks of radioactivity are detected. The first peak begins at tube 9-10 and trails off by 13. A second peak containing free ^{125}I begins at about tube 19. The iodinated rat PRL is contained in the first peak. About 20-40 % of the total radioactivity appears in the first peak. Usually the fractions high on the trailing shoulder of this peak (tubes 10-11) contain the most immunoactive and least damaged rat PRL. These fractions were stored at 4° C until repurified, not more than 14 days later, for rat PRL RIAs.

Preparation of the Sephadex column

A 1.5 x 30 cm chromatography column (Pharmacia Fine Chemical) is used for the preparation of the Sephadex column. The column is filled with Sephadex G-50 for

purification or Sephadex G-100 for repurification, previously equilibrated 48 hr with 0.05 M barbiturate buffer, pH 8.6, at room temperature, to a height of approximately 25 cm. The column is coated with 1 ml of 30 % BSA solution and washed thoroughly with the barbiturate buffer.

Repurification

Procedures

Immediately prior to RIAs, an aliquot of the iodinated rat PRL is applied to the Sephadex G-100 column. The column is then eluted with 0.05 M barbiturate buffer, pH 8.6, and fractions of 1.5 ml are collected in 18 x 150 mm glass culture tubes rinsed with 1 % BSA buffer.

Results

Two peaks of radioactivity were detected. The first peak begins at tubes 8-9 and trails off by tubes 21-23. A second peak containing free ^{125}I begins at about tubes 22-24. The iodinated rat PRL is contained in the first peak. Usually the fractions high on the trailing shoulder of this peak (tubes 15-17) contain the most immunoreactive and least damaged rat PRL. These fractions are pooled and diluted with 1 % BSA buffer such that 100 μl contains 10,000 to 12,000 cpm. This final solution is used for rat

prolactin radioimmunoassay.

Radioimmunoassay

- 1) Frozen samples (cultured media containing PRL) are thawed and diluted with 1 % BSA buffer such that a 100 μ l aliquot of the final solution contains 0.3125 to 10 ng of PRL.
- 2) Reactants and buffers are added into each 10 x 75 mm disposable test tube in the following sequence:
 - a) 0.2 ml of 1 % BSA buffer, or in case of a standard curve, 0.1 ml of 1 % BSA buffer + 0.1 ml of uncultured medium diluted the same way as the samples.
 - b) 0.1 ml of diluted samples, or in case for a standard curve, 0.1 ml of 1 % BSA buffer containing 0.3125 to 10 ng of the reference PRL.
 - c) 0.1 ml of the antiserum,
This antiserum is prepared just prior to RIAs by thawing and diluting the stored antiserum to 1 : 125 with 3 % normal rabbit serum buffer.
 - d) 0.1 ml of the iodinated and repurified PRL.
- 3) Agitate the tubes containing a total of 0.5 ml reactants and buffers on a vortex stirrer. Then, incubate for 21 hr at room temperature.
- 4) At the end of the incubation, add 1 ml of charcoal-dextran buffer into each tube to remove any free PRL.

Agitate the tubes on a vortex stirrer.

- 5) Centrifuge the tubes at 1,000 x g for 30 min at 4°C. The supernatant is suctioned and trapped into a radioactive waste container using an aspirator.
- 6) The precipitate is counted in a well type gamma spectrometer.
- 7) A standard curve is constructed on semi-logarithmic paper from B/Bo^a values of various concentrations of the reference preparation. The unknown amounts read directly from the curve, or indirectly by using a computer program. Our computer program transforms the raw data to arcsine, probit, and logit functions, and calculates a best fit line. This line is employed to calculate the amounts of PRL from samples. Results are expressed as ng of the reference preparation (NIAMD-Rat PRL-RP-1) per 0.1 ml of samples.

^a B/Bo represents a ratio of the radioactivity of PRL bound to the antiserum in presence of unlabeled PRL to that in absence of unlabeled PRL.

Preparations of buffers

1) 0.5 M sodium phosphate buffer (pH=7.6)

$\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$	6.9	Gm
$\text{Na}_2\text{HPO}_4 \cdot 12 \text{H}_2\text{O}$	17.9	Gm
H_2O	ad	100 ml

0.05 M sodium phosphate buffer (pH=7.6) is prepared by diluting the 0.5 M buffer to 1:10 with H_2O .

2) 3 % normal rabbit serum buffer (pH=7.6)

NaCl	0.82	Gm
EDTA	0.37	Gm
0.5 M NaH_2PO_4	0.24	ml
0.5 M Na_2HPO_4	1.76	ml
Rabbit serum ^a	3	ml
H_2O	ad	100 ml

pH is adjusted using 2N NaOH.

3) 1 % bovine serum albumin phosphate buffer (pH=7.6)

NaCl	8.2	Gm
Na_2EDTA	3.7	Gm
0.5 M NaH_2PO_4	2.4	ml
0.5 M Na_2HPO_4	17.6	ml
30 % BSA ^b	33.4	ml
H_2O	ad	1000 ml

pH is adjusted using 2 N NaOH.

4) 0.05 M sodium barbiturate buffer (pH=8.6)

Sodium barbiturate		8.907	Gm
Barbital		1.253	Gm
H ₂ O	ad	1000	ml

5) Dextran-coated charcoal buffer

Dextran T 70 ^c		1	Gm
Carbon Decolorizing Alkaline Norite-A ^d		10	Gm
Working solution	ad	400	ml

Working solution (pH=7.6)

Stock solution		100	ml
NaCl		8.5	Gm
H ₂ O	ad	2000	ml

pH is adjusted using 0.1 N HCl.

Stock solution

Sodium acetate		9.714	Gm
Sodium barbiturate		14.714	Gm
H ₂ O	ad	500	ml

^a Gland Island Biological Co.

^b Reheis Chemical Co.

^c Pharmacia Fine Chemical Co.

^d Fisher Scientific Co.

APPENDIX C

1. Calculation of $(^*P/P)_g - (^*P/P)_m$

$$(^*P/P)_g = \frac{K k_1/k_2 (1-e^{-k_2 t})}{k_1/k_2 (1-e^{-k_2 t}) + P_0 e^{-k_3 t}}$$

$$(^*P/P)_m = \frac{K [k_1 t - k_1/k_2 (1-e^{-k_2 t})]}{k_1 t - k_1/k_2 (1-e^{-k_2 t}) + P_0 (1-e^{-k_3 t})}$$

After subtraction,

$$\begin{aligned} \text{Denominator} &= [k_1/k_2 (1-e^{-k_2 t}) + P_0 e^{-k_3 t}] \\ &\quad \times [k_1 t - k_1/k_2 (1-e^{-k_2 t}) + P_0 (1-e^{-k_3 t})] \end{aligned}$$

$$\text{since, } k_1 t - k_1/k_2 (1-e^{-k_2 t}) > 0 \quad (t > 0)$$

denominator is always greater than 0, if t is positive.

$$\text{Numerator} = P_0 k_1/k_2 (1 - e^{-k_2 t} - k_2 e^{-k_3 t} t)$$

Suppose $k_2 = k_3$,

$$\text{If } f(t) = 1 - e^{-k_2 t} - k_2 e^{-k_2 t} t$$

$$\text{then } f'(t) = k_2^2 e^{-k_2 t} t > 0$$

Since $f'(t) > 0$ and $f(0) = 0$,

numerator is always positive, if t is positive.

Thus, $(^*P/P)_g > (^*P/P)_m$, if $t > 0$ and $k_2 = k_3$.

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