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In Vivo And In Vitro Regulation Of Pituitary Follicle-stimulating Hormone (fsh) Secretion By 3alpha-hydroxy-4-pregnen-20-one (3hp): A Highly Sensitive And Specific Inhibitor

Patricia Helen Wood

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IN VIVO AND IN VITRO REGULATION OF PITUITARY FOLLICLE-STIMULATING HORMONE (FSH) SECRETION BY 3α-HYDROXY-4-PREGNEN-20-ONE (3HP): A HIGHLY SENSITIVE AND SPECIFIC INHIBITOR

by

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ABSTRACT

The effect of the gonadal steroid, 3α-hydroxy-4-pregnen-20-one (3HP), in intact and castrate rats and in primary cultures of rat anterior pituitary cells and the possible interaction of 3HP with the signal transduction pathways of luteinizing hormone-releasing hormone (LHRH) was examined.

Intact or gonadectomized, male or female, prepubertal or adult rats treated with 3HP (0-200 ug/100 g BW; 1-4 days) showed a selective reduction in serum FSH levels (25%-60%) without any apparent effect on serum LH levels. The FSH-suppressing effect of 3HP was found to be dose-related, suggesting that 3HP action may be physiological. 3HP was effective in young male rats (10 d) and gonadectomized adult male rats, where 3HP showed its greatest suppression of serum FSH. Comparison of the effect of 3HP with that of some of its metabolites or other gonadal steroids showed that only 3HP had a selective FSH-suppressing effect.

In vitro, 3HP selectively inhibited the basal and LHRH-induced secretion of FSH, with no effect on LH secretion, in cells from both male and female rats. Inhibition of FSH secretion by 3HP was dose-dependent with the doses as low as 10^{-16} to 10^{-14} M exhibiting significant FSH-suppressing activity (by 35%). The cell content of FSH was unaffected by 3HP suggesting that synthesis, as well as release, of FSH is depressed by 3HP. As in the in vivo studies, none of the 18 other steroids tested, nor inhibin, had a similar selective FSH-suppressing action. It was concluded that FSH suppression by 3HP is specific to the structure of this molecule and that even minor alterations in the structure result in a loss of activity.

The effect of 3HP on the calcium signal and protein kinase C (PKC)

activation produced by LHRH was examined in vitro. In experiments employing the calcium ionophore, A23187, FSH secretion, but not LH secretion, was stimulated beyond the level produced by LHRH alone. Furthermore, although stimulated by calcium, it appears that FSH, but not LH, secretion is relatively calcium-independent and will occur in the absence of elevated calcium as revealed by treatment with the calcium channel antagonists, verapamil and nifedipine. The evidence suggests that 3HP acts to modify the FSH response to the calcium signalling pathway and that 3HP action on LHRH-induced FSH secretion might occur via an increase in the requirement of FSH secretion for calcium. Inhibition of PKC activity does not mimic the effect of 3HP. However, the inhibition of FSH release brought about by 3HP can be lessened by the activation of PKC. The activity of 3HP may also be due in part to down-regulation of the FSH gene. These studies have provided insight into the mechanism of 3HP action and further studies may help to completely elucidate this mechanism.

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Finally, thank-you for all your patient support and just for being there, Andy. The trials are over and the future is ours to share.

In loving memory of Anne Wood

whose spirit and determination live on forever

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ABBREVIATIONS

3βHP	3β-Hydroxy-4-pregnen-20-one
3HP	3α-Hydroxy-4-pregnen-20-one
17β-Estradiol	1,3,5(10)-Estratriene-3,17β-diol; estrogen
17α-Hydroxyprogesterone	17α-Hydroxy-4-pregnene-3,20-dione
A23187	Calimycin; calcium ionophore
ANOVA	Analysis of variance
B	Specific bound radioactivity (sample)
B_o	Total specific bound radioactivity
BSA	Bovine serum albumin
BW	Body weight
C_{19}	19-Carbon steroid; androstene or androstane
C_{21}	21-Carbon steroid; pregnene or pregnane
cm	Centimeter
CO_2	Carbon dioxide
cpm	Counts per minute (radioactivity)
Dihydrotestosterone	17β-Hydroxy-5α-androstan-3-one
EDTA	Ethylenediaminetetraacetic acid
FSH	Follicle-stimulating hormone
g	Gram
G	Gauge
GABA	Gamma-aminobutyric acid
GX	Gonadectomized
H-7	Protein kinase C inhibitor
hr	hour
^{125}I	Iodine-125 (radioisotope)
IgG	Immunoglobin G
Kd	Kilodaltons
KRP	Kreb's ringer phosphate
LH	Luteinizing hormone
LHRH	Luteinizing hormone-releasing hormone
M	Molar
M199	Medium 199; culture medium
mc	Millicurie
mg	Milligram
min	Minute
ml	Millilitre
mm	Millimeter
mM	Millimolar
ng	Nanogram
NIDDK	National Institute of Diabetes and Digestive and Kidney Diseases
nm	Nanometer
nM	Nanomolar
NSB	Nonspecific binding
P	Total culture protein
PBS	Phosphate buffered saline
PFF	Porcine follicular fluid
PFF-I	Porcine follicular fluid inhibin
PKC	Protein kinase C
Progesterone	4-Pregnen-3,20-dione
RIA	Radioimmunoassay
RNA	Ribonucleic acid

RP-1	Reference preparation 1 (NIDDK)
RP-2	Reference preparation 2 (NIDDK)
s.c.	Subcutaneous
SC-9	Protein kinase C activator
SCM	Sertoli cell culture medium
sec	Second
SEM	Standard error of the mean
Testosterone	17β-Hydroxy-4-antrosten-3-one
U	Unit
µg	Microgram
µl	Microlitre
µM	Micromolar
x g	Times gravity

INTRODUCTION

It is well established that gonadal steroids play an important role in the differentiation, development and regulation of the reproductive systems of mammals. The interaction of neural signals, hypothalamic releasing factors, pituitary hormones and gonadal steroids provide a fine control of all reproductive processes. The current understanding is that this system (neural-hypothalamic-pituitary-gonadal axis) is very complex and that there may be a number of factors involved that are not yet defined. This thesis attempts to add to the body of knowledge concerning reproductive controls in the mammal and to define a role in these processes for a relatively novel gonadal steroid, 3α-hydroxy-4-pregnen-20-one (3HP).

1.1 THE HYPOTHALAMIC-PITUITARY GONADAL AXIS

1.1.2 The Endocrine Hypothalamus

The hypothalamus is located at the base of the mammalian brain surrounding the third ventricle (Paxinos and Watson, 1986). The releasing factor for gonadotropic hormones is synthesized, and released in a pulsatile manner (Bourguinon *et al*, 1987), by neurons within the medial basal hypothalamus and transported to the median eminence for storage (Hadley, 1984). Luteinizing hormone-releasing hormone (LHRH) is a decapeptide whose structure was first elucidated by Baba, Matsuo and Schally (1971). LHRH is transported via the hypophysial portal vascular system from the median eminence (Schally *et al*, 1977) to the anterior pituitary where it greatly enhances the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) in numerous mammals

including rats, rabbits, pigs, horses and humans, among others, regardless of the route of administration (Schally et al, 1976).

1.1.2 The Anterior Pituitary

The adenohypophysis or anterior pituitary gland is located in a pocket of the sphenoid bone at the base of the brain (Paxinos and Watson, 1986) and communicates with the median eminence of the hypothalamus via the hypophysial portal system (Schally et al, 1977). Numerous tropic hormones are synthesized by the cells of the anterior pituitary including LH and FSH produced by gonadotropes (Hadley, 1984). Gonadotropes mature early in the rat (in the first week of life) and 75% of gonadotropes contain both FSH and LH secretory granules while 14.2% contain LH, and 11% contain FSH secretory granules only (Childs et al, 1981). These secretory granules are seen to be depleted after stimulation of anterior pituitary gonadotropes by LHRH (Nakamura et al, 1985). Simultaneous measurement of LHRH and serum FSH and LH in intact and castrate rats has shown that circulating levels of FSH and LH are highly correlated to the release of LHRH (Levine and Duffy, 1988).

1.1.3 The Gonad

The male gonad, or testis, consists of two compartments; (1) the seminiferous tubules which produce sperm in an environment carefully controlled by Sertoli cells and (2) the interstitium where the cells of Leydig produce androgens for the maintenance of the male reproductive tissues (Burgos et al, 1970). Pituitary gonadotropins (FSH and LH) regulate testicular function. Luteinizing hormone activates Leydig cells (Solano et al, 1988) and has a positive effect on the production of

androgens, particularly testosterone (17β-hydroxy-4-androsten-3-one), by these cells (Eik-Nes, 1975). LH action in the interstitium results in increased activity of 3β-hydroxysteroid dehydrogenase, an important enzyme in testicular androgen synthesis (Shikita and Hall, 1967; Wiebe, 1978a). Traditionally, testosterone has been thought to be the prime regulator of gonadotropin secretion, particularly LH, in male mammals (Zanisi et al, 1973).

Follicle-stimulating hormone binds specifically to Sertoli cells, resulting in a stimulation of spermatogenesis (Means et al, 1980; Means, 1975 provides a good review). Evidence has accumulated which indicates that rat Sertoli cells not only "nurse" the developing spermatozoa but also are capable of producing hormones themselves. In 1970, Lacy and Pettit definitively described steroidogenic activity in seminiferous tubules. Sertoli cells from young rats were further shown to metabolize C_{19} steroids under FSH stimulation (Welsh and Wiebe, 1976; Welsh and Wiebe, 1978), to metabolize pregnenolone (3β-hydroxy-5-pregnen-20-one) and progesterone (4-pregnene-3,20-dione) (Wiebe, 1978b; Tilbe and Wiebe, 1981) and to synthesize steroids de novo from acetate (Wiebe and Tilbe, 1979). The primary products of rat Sertoli cell progesterone metabolism are C_{21} steroids (see Wiebe, 1985 and Wiebe et al, 1988 for reviews). Recent work has shown that inhibin (a polypeptide hormone) is also produced in seminiferous tubules under the influence of FSH and plays a role in the feedback inhibition of pituitary FSH secretion (Au et al, 1986; McLachlan et al, 1987; deKretser and Robertson, 1989).

Gonadotropins from the anterior pituitary regulate ovarian function in female mammmals. FSH is responsible for the recruitment and growth of follicles in the ovary (Hirshfield, 1979). The primary stimulus of

steroidogensis in the ovary is luteinizing hormone. LH binds to thecal cells in ovarian follicles and stimulates the production of androgens from cholesterol (Fortune and Armstrong, 1979; Gore-Langton and Armstrong, 1988). Thecal androgens are further converted to 17β-estradiol (1,3,5(10)-estratriene-3,17β-diol) by granulosa cells under the influence of FSH early in the follicular stage of the ovarian cycle (Dorrington and Armstrong, 1979; Gore-Langton and Armstrong, 1988). It is 17β-estradiol which provides negative feedback regulation of gonadotropin secretion in the female, while progesterone produced by the granulosa cells later in the cycle, causes a surge in gonadotropins (Gore-Langton and Armstrong, 1988). There is also evidence that ovarian follicles produce inhibin which has a negative effect on FSH secretion (Ling et al, 1985; Fukuda et al, 1986; Martin et al, 1986).

Figure 1 and Figure 2 summarize the function of the hypothalamic-pituitary-gonadal axis in the male and female mammal, respectively.

1.2 REGULATION OF GONADOTROPIN SECRETION IN VIVO

Many studies have been performed over the years to discern the mechanisms by which reproductive function is regulated. Experiments on intact and gonadectomized animals have shown that gonadal steroids play a major role in regulating gonadotropin secretion and that there are some differences in regulatory mechanisms between males and females.

1.2.1 Gonadotropin Secretion in the Male

The concentration of gonadotropic hormones in the blood of male rats is controlled by feedback mechanisms involving the gonads. Serum LH levels normally range from 25-100 ng (NIDDK rat LH reference preparation

Figure 1: Hypothalamic-pituitary-gonadal axis in the male rat. Luteinizing hormone-releasing hormone (LHRH) is synthesized in, and secreted from, the endocrine hypothalamus of the brain. LHRH travels to the pituitary via the hypophysial portal (vascular) system where the gonadotropes are stimulated to secrete luteinizing hormone (LH) and follicle-stimulating hormone (FSH) into the bloodstream. LH stimulates the production of androgens within the Leydig cells of the testis (particularly testosterone) which in turn have a negative feedback effect on the hypothalamus and pituitary to decrease the release of LH. FSH binds to Sertoli cells in the testis and stimulates spermatogenesis, steroid metabolism and the production of the polypeptide, inhibin. Current views are that inhibin secreted into the seminal fluid and subsequently taken up into the bloodstream is responsible for negative feedback control of FSH release in the male.

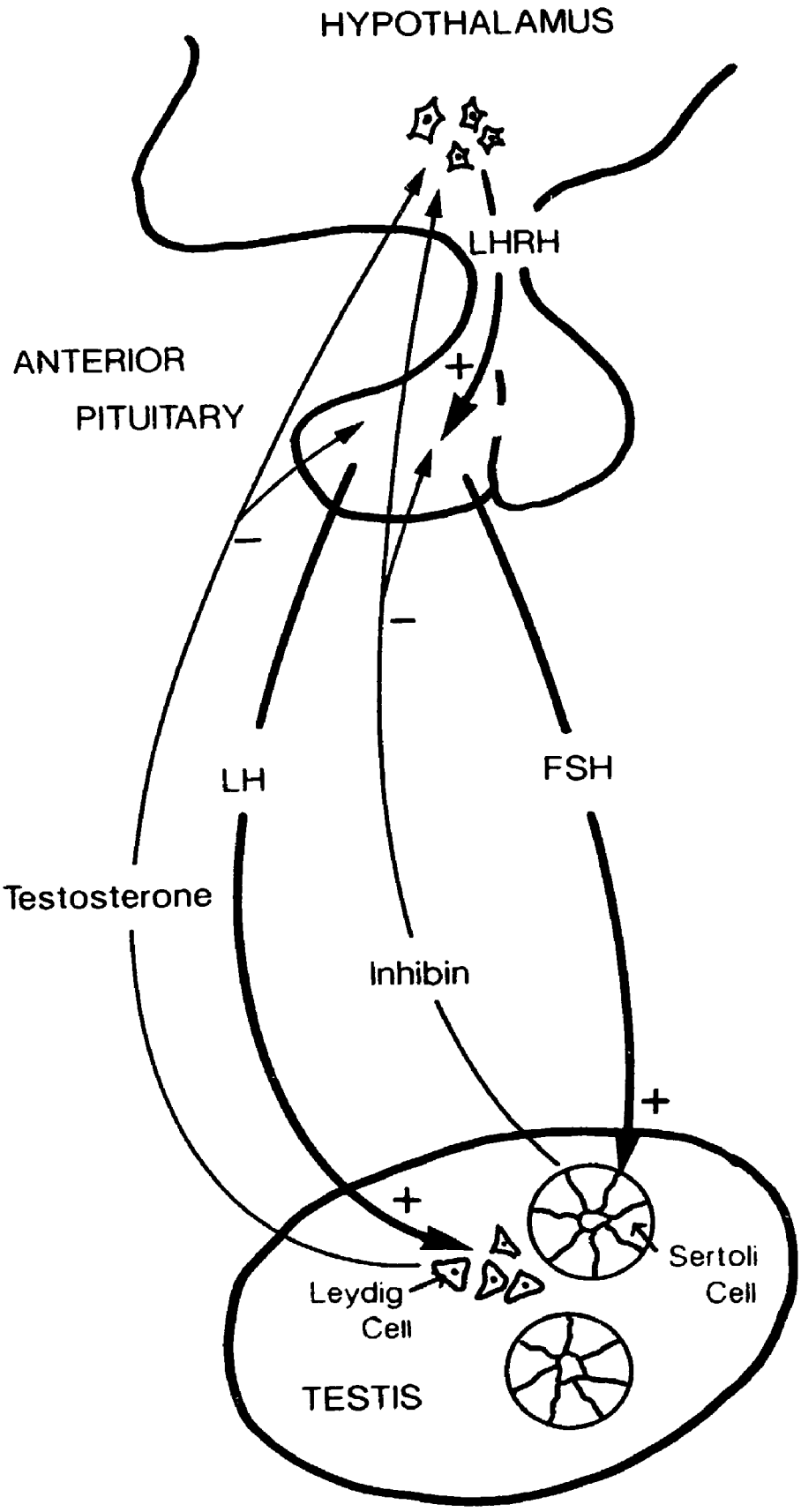
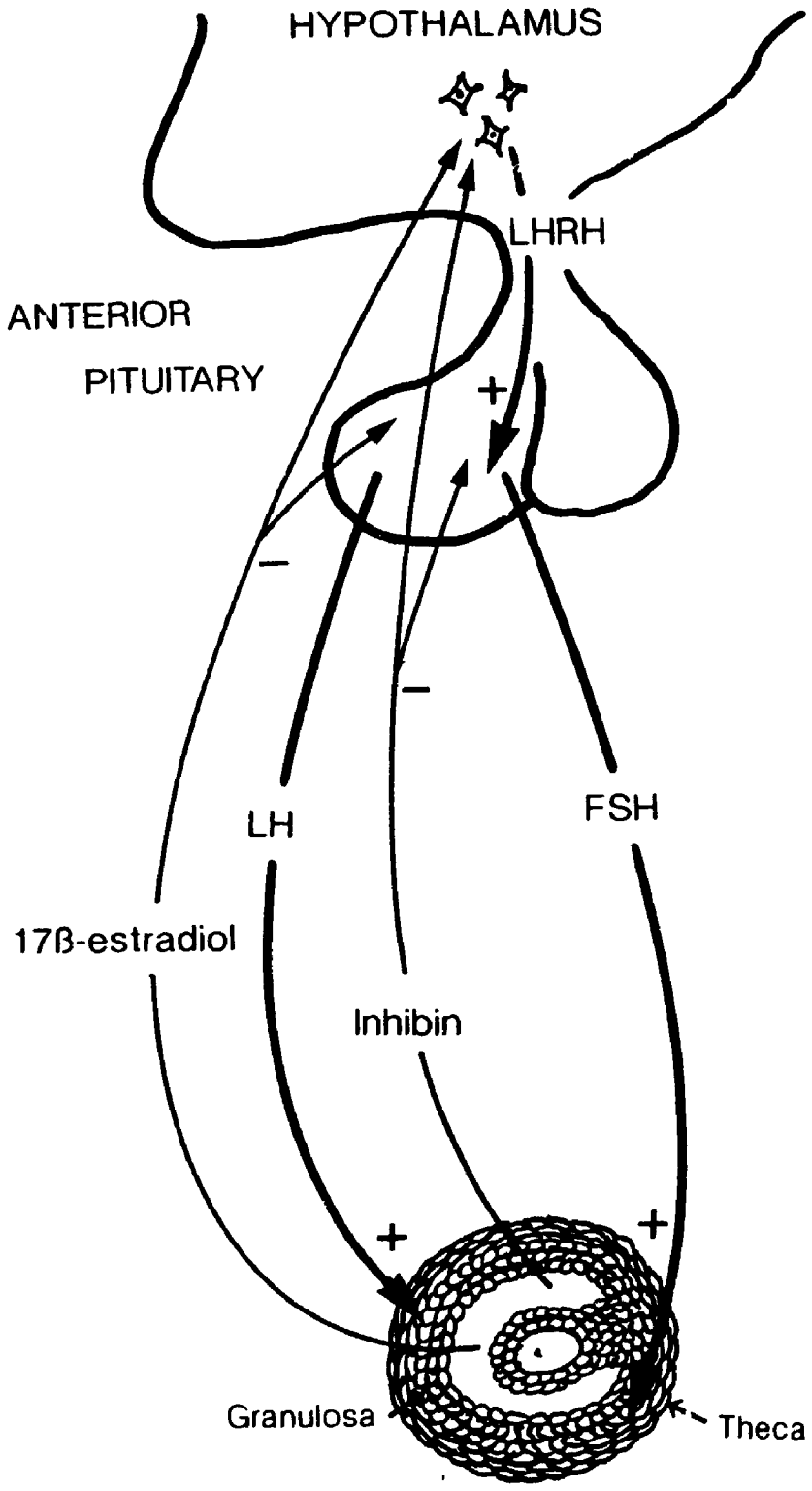

HYPOTHALAMUS
LHRH
ANTERIOR
PITUITARY
+
−
−
LH
FSH
Testosterone
Inhibin
+
+
Sertoli
Cell
Leydig
Cell
TESTIS

Figure 2: Hypothalamic-pituitary-gonadal axis in the female rat. Luteinizing hormone-releasing hormone (LHRH) is synthesized in, and secreted from, the endocrine hypothalamus of the brain. LHRH travels to the pituitary via the hypophyseal portal (vascular) system where the gonadotropes are stimulated to secrete luteinizing hormone (LH) and follicle-stimulating hormone (FSH) into the bloodstream. LH stimulates the production of androgens within the thecal layer of ovarian follicles. FSH stimulates the granulosa cells to produce 17β-estradiol from the androgen precurors produced by the thecal cells, which has a negative feedback effect on the hypothalamus and pituitary to decrease gonadotropin release, from the thecal androgen precursers. Later in the ovarian cycle, granulosa cells produce progesterone which plays a role in the stimulation of the pre-ovulatory gonadotropin surge. Ovarian follicles also produce inhibin which is involved in the inhibition of FSH secretion from the anterior pituitary.



OVARIAN FOLLICLE

1; RP-1)/ml with usual values for adult animals of 60-100 ng/ml (Dohler and Wuttke, 1975; McLean et al, 1977). Serum FSH is somewhat higher, with average adult values of around 500 ng/ml (RP-1) (Dohler and Wuttke, 1975; Lorenzen and Ramaley, 1981; McLean et al, 1977). McLean and his co-workers (1977) also found that, in the adult male rat, serum FSH varies diurnally from a low of 400-800 ng/ml to a high of 1100-1300 ng/ml. Gonadectomy, or castration, of the male rat removes the source of hormones which negatively control gonadotropin secretion and results in 3-4 fold increases in serum LH and 2-3 fold increases in serum FSH (Lorenzen and Ramaley, 1981; Hiatt et al, 1987). Secretion of LHRH from the hypothalamus is also increased after gonadectomy and is highly correlated to the rise in serum gonadotropins following castration (Urbanski et al, 1988; Levine and Duffy, 1988).

Many factors are involved in the regulation of circulating gonadotropin levels in the male. Androgens (C_{19} steroids) are produced in considerable quantities by testicular Leydig cells (Eik-Nes, 1975) and play a major role in regulating the secretion of pituitary gonadotropins, particularly LH. Testosterone, when administered to the intact male rat, is capable of suppressing serum LH levels and has a somewhat lesser effect on serum FSH levels (Gray et al, 1980). A similar role for testosterone in the intact male rat was demonstrated by active immunization against testosterone, which results in increases in serum gonadotropins (Nieschlag et al, 1975). In the castrate rat, testosterone at high doses (^{1}300 µg/100 g body weight (BW)) can completely suppress the post-gonadectomy rise in serum gonadotropins (Eldridge et al, 1974; Summerville and Schwartz, 1981). Most studies show a parallel inhibition of gonadotropins with a preferential reduction of Lh secretion with

doses of testosterone in the 10-300 µg/100 g BW range (Gay and Dever, 1971; Dufy-Barbe and Franchimont, 1971; Swerdloff and Walsh, 1973).

Many steroids have been investigated using the gonadectomized male rat model. In the gonadectomized male rat, dihydrotestosterone (17β-hydroxy-5α-androstan-3-one) selectively inhibits LH secretion at doses of 20 or 70 µg/100 g BW when administered for 4 days (Demoulin et al, 1973; Swerdloff et al, 1973) and gives a parallel inhibition of LH and FSH secretion at higher doses, with the emphasis on depressing LH secretion (Demoulin et al, 1973; Swerdloff et al, 1973; Eldridge et al, 1974; Verjans and Eik-Nes, 1977). It appears that dihydrotestosterone is more potent than testosterone in reducing the secretion of gonadotropins from the pituitary (Loseva et al, 1980). Of considerable interest is the fact that extremely low doses of dihydrotestosterone (less than 10 µg/100 g BW/day) increase basal FSH secretion while LH levels are suppressed (Mittler et al, 1981; Karanth et al, 1984) and increase the sensitivity of pituitary LH to LHRH induction while FSH sensitivity to LHRH is reduced (Karanth et al, 1984). Furthermore, 50 ng of crystalline testosterone implanted in the lateral septum of male rats has a positive effect on the secretion of LH and FSH (Carreras et al, 1987).

Several other androgens are implicated in the regulation of pituitary function. The most studied of these is 5α-androstane-3α,17β-diol which adequately reduces serum LH in the gonadectomized male rat at doses as low as 20 µg/100 g BW but does not suppress FSH secretion until higher doses of 1000 µg/100g BW are employed (Demoulin et al, 1973; Swerdloff et al, 1973; Verjans and Eik-Nes, 1977). Among androgens shown to selectively reduce serum LH levels in the gonadectomized male rat

are: 5α-androstane-3β,17β-diol (Swerdloff et al, 1973; Loseva et al, 1980); 4-androstene-3,17-dione; 4-androstene-3α,17β-diol; 5-androstene-3α,17β-diol and 3β-hydroxy-5α-androstan-17-one (epiandrosterone) (Swerdloff et al, 1973).

There is considerable evidence that 17β-estradiol is produced in the male gonad and may play a role in regulating pituitary secretion (Eik-Nes, 1975; van der Molen et al, 1973; Winter and Troen, 1985). Studies show that, similar to testosterone and dihydrotestosterone, 17β-estradiol treatment gives rise to a parallel inhibition of serum LH and FSH in the gonadectomized male rat, with LH levels depressed more than those of FSH (Gay and Dever, 1971; Swerdloff and Walsh, 1973; Swerdloff et al, 1973; Eldridge et al, 1974). However, Demoulin et al (1973) showed that a low dose of 17β-estradiol (4 x 0.085 μg/rat) results in a considerable increase in serum FSH. In similar studies, active immunization of intact male rats against 17β-estradiol showed a clear role for 17β-estradiol in the negative feedback control of LH secretion (Nieschlag et al, 1975) while treatment of healthy men with the 17β-estradiol antagonist, clomiphene citrate, results in a sharp rise in serum LH levels with a lesser effect on serum FSH levels (Winter and Troen, 1985). The authors of these experiments suggest that 17β-estradiol endogenously acts to restrain the release of hypothalamic LHRH.

The testis has the capacity to produce 21-carbon steroids (pregnenes/pregnanes;Lacy and Pettit, 1970; Wiebe, 1985). In the gonadectomized male rat, progesterone has no effect on the secretion of gonadotropins from the adenohypophysis (Swerdloff et al, 1973; Eldridge et al, 1974). Swerdloff et al (1973) also a tested a number of other C_{21}

steroids in the castrate male rat including 17α-hydroxy-4-pregnene-3,20-dione (17α-hydroxyprogesterone), 20α-hydroxy-4-pregnen-3-one and 3β-hydroxy-5-pregnen-20-one (pregnenolone) among others, all of which have no effect on circulating levels of gonadotropins. McPherson and Mahesh (1982) found that a "surge" of LH and FSH in the gonadectomized male rat could be produced by 100 μg/100 g BW progesterone after 4 days of estrogen-priming (2 μg/100 g BW). Evidence also exists to suggest that adrenal C_{21} steroids are involved in the regulation of pituitary gonadotropin secretion. Cortisol (11β,17α,21-trihydroxy-4-pregnene-3,20-dione) suppresses the LH, but not the FSH, response to LHRH in gonadectomized male rats (Ringstrom and Schwartz, 1985) and adrenalectomy of prepubertal male rats decreases serum FSH and the FSH response to LHRH induction (Nazian, 1982). Debeljuk and deRettori (1982) and Motta et al (1981) provide comprehensive reviews of the effects of gonadal hormones on the secretion of gonadotropins and conclude that the regulation of FSH secretion is not completely understood nor explained by the effects of gonadal steroids.

Evidence has existed since the 1930's which suggests the involvement of the seminiferous tubules in the regulation of FSH secretion (McCullagh, 1932). This regulator of FSH was shown to be a product of rat Sertoli cells in the early 1970's (VanThiel et al, 1972; Krueger et al, 1974) and later in the decade was dubbed "inhibin" (a name first proposed by McCullagh) for its FSH-suppressing property (Steinberger and Steinberger, 1976; Keogh et al, 1976). Inhibin is a polypeptide of molecular weight 32,000 daltons containing two subunits joined by a disulphide bridge (Ling et al, 1985; Fukuda et al, 1986). deKretser and Robertson (1989) and Vale et al (1988) provide good

reviews on the isolation and physiology of inhibin. Numerous studies have shown that inhibin suppresses serum FSH levels in intact (Sheth et al, 1980; Sheth and Vijayalakshmi, 1981; Lipner and Dhanarajan, 1984) and gonadectomized (Shahmanesh et al, 1980; Summerville and Schwartz, 1981; Thomas and Nikitovitch-Winer, 1984) male rats. Most studies suggest that inhibin works at the pituitary level to regulate the synthesis and secretion of FSH (and perhaps LH) (Lipner and Dhanarajan, 1984; Shahmanesh et al, 1980). Morris and Azmatullah (1982) have shown that inhibin does not modulate the pituitary FSH response to LHRH in the castrate rat while other investigators have suggested that inhibin accounts for perhaps only 20-80% of FSH regulation and that another gonadal product may be involved (Summerville and Schwartz, 1981; Decker et al, 1981; Sheth and Vijayalakshmi, 1981). Finally, inhibin production and Sertoli cell content of inhibin is maximal in the prepubertal rat (8-21 days) when FSH levels are low, but then decline while FSH levels rise, suggesting that inhibin may be an important regulator of FSH only in the prepubertal male rat (Sheth et al, 1980; Au et al, 1986; Ultee-vanGessel and deJong, 1987; Rivier et al, 1988).

Table 1 summarizes the effect of gonadal hormones on the secretion of pituitary gonadotropins in the male rat.

1.2.2 Gonadotropin Secretion in the Female: A Brief Review

Gonadotropin secretion in the female has been much more extensively studied than in the male and is therefore better understood. A brief review is presented here for comparative purposes. LH and FSH secretion in the female is cyclic in nature with a peak in the secretion of both gonadotropins occuring just prior to ovulation. Normal serum LH levels

Table 1: Summary of the effects of gonadal hormones on the in vivo secretion of gonadotropins in the male rat.

Hormone[1]	INTACT FSH	INTACT LH	GX FSH	GX LH	References
Testosterone	↓	↓↓	↓	↓↓	Gray et al (1980); Nieschlag et al (1975); Eldridge et al (1974); Summerville and Schwartz (1981); Gay and Dever (1971); Dufy-Barbe and Franchimot (1971); Swerdloff and Walsh (1973)
50 ng in lateral septum	↑	↑	ns	ns	Carreras et al (1987)
Dihydrotesosterone	ns	ns	↓	↓↓	Demoulin et al (1973); Swerdloff et al (1973); Eldridge et al (1974); Verjans and Eik-nes (1977)
low doses	↑	↓	↓	↑	Mittler et al (1981); Karanth et al (1984)
5α-Androstane-3α,17β-diol	ns	ns	↓	↓↓	Demoulin et al (1973); Swerdloff et al (1973); Verjans and Eik-nes (1977)
Other androgens	ns	ns	--	↓	Swerdloff et al (1973); Loseva et al (1980)
17β-Estradiol	↓	↓↓	↓	↓↓	Gay and Dever (1971); Swerdloff et al (1973); Swerdloff and Walsh (1973); Eldridge et al (1974); Nieschlag et al (1975); Winter and Troen (1985)
low dose	ns	ns	↑	--	Demoulin et al (1973)
Progesterone	ns	ns	--	--	Swerdloff et al (1973); Eldridge et al (1974)
Progesterone + 17β-estradiol	ns	ns	↑	↑	McPherson and Mahesh (1982)
Glucocorticoids	↑	--	↑	↓	Nazian (1982); Ringstrom and Schwartz (1985)
Inhibin	↓↓	--	↓↓ [--]	--	Sheth et al (1980); Lipner and Dhanarajan (1984); Shahmanesh et al (1980); Thomas and Nikitovich-Winer (1984); [Morris and Azmutullah (1982)]

Table 1: continued

[1] see text for details of doses, etc.
GX, gonadectomized
ns, not studied
--, no effect
arrows indicate relative increase/decrease in gonadotropin secretion

are 25-60 ng/ml (RP-1) in the prepubertal female , around 50 ng/ml in metestrous and diestrous adults and between 1200-1800 ng/ml in the proestrous and estrous adult (Dohler and Wuttke, 1975). The level of circulating FSH is very high in the infantile female (800-900 ng/ml), lower in the prepubertal female (150-200 ng/ml), with similar levels in metestrus and diestrus adults, and surges to around 400 ng/ml in proestrus and estrus adults (Dohler and Wuttke, 1975). Gonadectomy of female rats results in 4-fold increases in FSH secretion and an approximately 3-fold increase in LH secretion (Lorenzen and Ramaley, 1981; Hiatt et al, 1987).

The ovarian steroids, 17β-estradiol and progesterone are the primary regulators of gonadotropin secretion in the female rat. 17β-estradiol inhibits the release of gonadotropins in ovariectomized female rats (Motta et al, 1981; McPherson et al, 1975; Matt et al, 1986) but potentiates the pituitary response to LHRH (Debeljuk and deRettori, 1982). If 17β-estradiol administration is conconcurrent with, or followed by, treatment with progesterone, a surge in the release of gonadotropins is initiated (Fink and Henderson, 1977; McPherson et al, 1975) probably via 17β-estradiol induction of progesterone receptors in the hypothalamus and pituitary (Attardi, 1984; Mahesh and Muldoon, 1987; Calderon et al, 1987). The 5α-reduced metabolite of progesterone, 5α-pregnane-3,20-dione, produced in the pituitary and hypothalamus, appears to be responsible for the surge in FSH secretion in the estrogen-primed ovariectomized female rat (Karavolas et al, 1976, Motta, 1982; Motta et al, 1981; Murphy and Mahesh, 1984a) while 3α-hydroxy-5α-pregnan-20-one (another progesterone metabolite) appears to be responsible for the similar surge in LH (Motta et al, 1981; Motta, 1982; Murphy and Mahesh,

1984b). Several C_{19} steroids with a 4-ene configuration were shown to have the same abilities as estrogen (Kraulis et al, 1978a) and several adrenal C_{21} steroids were shown to have the same effect as progesterone in inducing gonadotropin surges (Kraulis et al, 1978). Inhibin is produced in rat ovarian follicles (Vale et al, 1988; deKretser and Robertson, 1989) and possesses FSH-suppressing activity which is both specific and age-dependent (Hermans et al, 1980; McLachlan et al , 1987).

1.3 REGULATION OF GONADOTROPIN SECRETION IN VITRO

The ongoing modulation of gonadotropin secretion in the live animal is difficult to assess. The models used in the studies outlined in the previous section must take into account that many other factors besides the pituitary (eg. the central nervous system) may have an influence on the results of the various in vivo experiments. Current research often relies on an in vitro system, where anterior pituitary cells (and sometimes hypothalami) from rats are maintained in short-term primary cultures to examine the possible effects of hormones under more controlled experimental conditions.

1.3.1 Basal Gonadotropin Secretion

Gonadotropes in primary cultures of anterior pituitary cells tonically secrete FSH and LH and retain the ability to respond to gonadal steroids or other hormones. The effect of androgens in this system is well documented. Most studies agree that concentrations of testosterone in the medium of 1 nM or greater will increase the basal release of FSH and not affect LH release (Steinberger and Chowdhury,

1977; Leveque and Grotjan, 1982; Kennedy and Chappel, 1985; Campen and Vale, 1988) although an older report by Tang and Spies (1975) suggests that 10 nM testosterone increases basal LH secretion as well. Denef et al (1980) reported that concentrations of testosterone up to 50 nM have no effect on basal gonadotropin secretion. Evidence also suggests that testosterone enhancement of FSH release involves increased FSH synthesis (Kennedy and Chappel, 1985). Dihydrotestosterone, the 5α-reduced metabolite of testosterone, will also enhance basal FSH secretion without affecting LH secretion at doses of 1 nM or greater (Leveque and Grotjan, 1982; Massicotte et al, 1984a; Massicotte et al, 1984b; Campen and Vale, 1988) and this effect, too, involves the stimulation of FSH synthesis (Leveque and Grotjan, 1982). Similar studies by Schwaninger et al (1987) showed no effect of dihydrotestosterone on basal gonadotropin secretion when used in cultures at concentrations between 1 nM and 100 mM which agrees with a report by Denef et al (1980). Campen and Vale (1988) demonstrated that androstenedione (4-androstene-3,17-dione) behaves similarly to testosterone and dihydrotestosterone, increasing the basal release of FSH by cultured pituitary cells.

The in vitro secretion of gonadotropins is also regulated by 17β-estradiol. 17β-Estradiol (10 nM) stimulates the basal secretion of both FSH and LH (Tang and Spies, 1975; Debeljuk et al, 1978; Steinberger and Chowdhury, 1977; Massicotte et al, 1984a; Massicotte et al, 1984b). Miller and Wu (1981) showed a similar effect of 1 nM 17β-estradiol in cultures of rat pituitary cells, but noted that similar treatment of cultured sheep, pig or cow adenohypophyseal cells results in decreased basal release of FSH. Miller and Wu (1981) also demonstrated that the

effect of 17β-estradiol involves an increase in FSH synthesis. Progesterone alone will augment the basal release of FSH at high concentrations (100 nM) (Massicotte et al, 1984a and 1984b; Leveque and Grotjan, 1982) but has no effect on the secretion of gonadotropins in cultured rat pituitary cells at lower (10 nM) doses (Tang and Spies, 1975). Leveque and Grotjan (1982) also indicated that FSH synthesis was stimulated by progesterone. Batra and Miller (1985) and Batra et al (1986) have shown that progesterone (100 nM) inhibits the secretion of FSH in ovine pituitary cells in culture. Many studies have used the technique of estrogen-priming to further elucidate the effect of progesterone on gonadotropin secretion since 17β-estradiol is known to induce the production of progesterone receptors in the pituitary and hypothalamus in vivo (Attardi, 1984; Calderon et al, 1987; Mahesh and Muldoon, 1987). Massicotte and colleagues (1984a and 1984b) have shown that pretreatment of cells with 10 nM 17β-estradiol considerably increases the release of both gonadotropins after treatment with 100 nM progesterone and that this effect is synergistic.

Other C_{21} steroids have been examined for their ability to modify the in vitro function of pituitary cells. Tang and Spies (1975) have shown that 10 nM 20α-hydroxy-4-pregnen-3-one will inhibit the basal secretion of FSH. The glucocorticoids, cortisol and corticosterone (11β,21-dihydroxy-4-pregnene-3,20-dione), inhibit the basal release of LH and stimulate the basal release of FSH when used at a concentration of 60 ng/ml or 600 ng/ml (Suter and Schwartz, 1985a). Synthesis of the gonadotropins was affected in a similar manner by the glucocorticoids.

The selective effect of inhibin on FSH secretion has been extensively studied in vitro. One of the first such studies indicated

that porcine follicular fluid (PFF) inhibin selectively reduces the release of FSH in cultured pituitary cells (Lagace et al, 1979). Massicotte et al (1984a) investigated the effects of inhibin from Sertoli cell culture medium (SCM) on gonadotropin secretion in rat anterior pituitary cells in culture. They found that SCM selectively inhibited basal FSH secretion in a dose dependent manner, but this inhibition could be overcome by the stimulatory effects of 10 nM dihydrotestosterone, 100 nM progesterone or 10 nM 17β-estradiol plus 100 nM progesterone. They (Massicotte et al, 1984b) achieved similar results with inhibin from granulosa cell culture medium. Similarly, Fukuda et al (1987) used purified 32 Kd inhibin from PFF and found that doses as low as 1 ng/ml selectively inhibited the secretion and synthesis of FSH and that this effect is mimicked by the protein synthesis inhibitor, cycloheximide. Purified 32 Kd inhibin from ovine rete testis fluid is also an effective inhibitor of in vitro FSH secretion at concentrations of 1 pM or greater and testosterone can not modify this effect (Campen and Vale, 1988). However some controversy still exists: purified 31 Kd inhibin from bovine follicular fluid (0.1 U/ml or more) depresses the secretion and synthesis of both FSH and LH in cultured pituitary cells (Farnworth et al, 1988).

1.3.2 LHRH-Induced Gonadotropin Secretion

Cultured anterior pituitary cells from rats retain the ability to respond to luteinizing hormone-releasing hormone (LHRH) with an increase in gonadotropin secretion (Liu and Jackson, 1984; Jinnah and Conn, 1985). Maximum stimulation of LH release in vitro can be attained with 10 nM LHRH (Drouin and Labrie, 1976; Pohl et al, 1987). More information

on the role of gonadal hormones in the regulation of pituitary gonadotropin secretion has been gleaned from studies which employ LHRH stimulation of gonadotropin secretion.

Most studies agree that testosterone at concentrations of 10 nM or more will enhance the FSH response (ie. FSH secretion in response) to LHRH (Tang and Spies, 1975; Leveque and Grotjan, 1982; Kennedy and Chappel, 1985) and increase the synthesis of FSH after LHRH stimulation (Leveque and Grotjan, 1982; Kennedy and Chappel, 1985). Drouin and Labrie (1976) found that the same dose (10 nM) of testosterone inhibits the LH response, and synthesis of LH, to 0.1 nM LHRH and, although the FSH response (i.e. secretion) to LHRH is unaffected by testosterone, the synthesis of FSH is stimulated. Denef et al (1980) showed that 0.5 to 50 nM testosterone inhibited both the FSH and LH response to LHRH. Only the study of Kao and Weisz (1976) disagrees with the concensus and claims that 1 μg/ml testosterone enhances the LH response and inhibits the FSH response to LHRH (in the form of hypothalamic extract). Dihydrotestosterone behaves similarly to testosterone in cultured pituitary cells; 10 nM concentrations of dihydrotestosterone increase the secretion and synthesis of FSH in response to LHRH (Massicotte et al, 1984a and 1984b; Leveque and Grotjan, 1982; Schwaninger et al, 1987). LHRH-induced LH secretion and synthesis is inhibited by dihydrotestosterone at doses of 0.1 nM or greater (Drouin and Labrie, 1976; Massicotte et al, 1984a and 1984b; Schwaninger et al, 1987). One study suggests that the LHRH-induced secretion of both FSH and LH is decreased by dihydrotestosterone at doses of up to 50 nM (Denef et al, 1980). Campen and Vale (1988) have found that, in their culture system, the androgens, testosterone, dihydrotestosterone and androstenedione (3

nM), inhibit the LHRH-induced secretion of LH and FSH. Girre et al (1980) found that 5α-androstane-3β,17β-diol inhibits the LHRH-induced release of FSH at doses of 100 ng/ml or greater.

17β-estradiol has a facilitating effect on gonadotropin secretion from cultured adenohypophyseal cells. Ten nM 17β-estradiol significantly increases FSH release in response to 0.3 nM LHRH (Massicotte et al, 1984a and 1984b) and enhances the LH response to 1 nM LHRH (Hsueh et al, 1979). Lagace et al (1980), Kotsuji et al (1988) and Liu and Jackson (1988) found a similar effect of 17β-estradiol on LHRH-induced FSH and LH secretion. Progesterone is more selective and only facilitates the LHRH-induced release of FSH. FSH release under the influence of various doses of LHRH is significantly enhanced by the addition of 100 nM progesterone to the incubation medium (Lagace et al, 1980; Leveque and Grotjan, 1982; Massicotte et al, 1984a and 1984b). In an interesting study, Kiesel et al (1987) showed that contraceptive progestins (eg. cyproterone, ethinodiol acetate and norethisterone) decrease both the LH and FSH response to LHRH. Pretreatment or simultaneous treatment of anterior pituitary cells in culture with 17β-estradiol (10 nM) and 100 nM progesterone greatly enhances the FSH stimulating effect of progesterone and even elicits a slightly greater LHRH-induced LH response than seen with progesterone alone (Lagace et al, 1980; Leveque and Grotjan, 1982; Massicotte et al, 1984a and 1984b). A good review of the early work on gonadal steroid effects on LHRH-induced gonadotropin secretion is given by Labrie et al (1978).

Glucocorticoids also influence the gonadotropin response to LHRH in vitro. Corticosterone decreases LH secretion in response to LHRH without affecting LH synthesis and increases the synthesis and secretion

of FSH in response to LHRH treatment (Suter and Schwartz, 1985; Kamel and Kubajak, 1987).

Inhibin, the putative regulator of FSH secretion, is not selective when it comes to LHRH-induced gonadotropin secretion. All studies agree that inhibin, regardless of its source, reduces the LHRH-induced synthesis and secretion of FSH and the release of LH (Lagace et al, 1979; Massicotte et al, 1984a and 1984b; Fukuda et al, 1987; Campen and Vale, 1988). This inhibitory effect, which is enhanced by the androgens (Campen and Vale, 1988) and opposed by the synergistic combination of 17β-estradiol and progesterone (Massicotte et al, 1984a and 1984b), occurs approximately 4 hr after treatment and is mimicked by cycloheximide (Fukuda et al, 1987). Several studies have recently shown that inhibin decreases the binding of, and probably the receptors for, LHRH in cultured pituitary cells and even decreases the ability of LHRH to up-regulate its own receptors (Wang et al, 1988; Wang et al, 1989). These last three studies suggest that inhibin acts via an inhibition of protein synthesis in pituitary cells. For good reviews of the current state of knowledge about inhibin see deKretser and Robertson (1989) or Vale et al (1988).

Table 2 summarizes the effect of steroid and gonadal hormones on the release of gonadotropins by cultured rat anterior pituitary cells.

1.4 HORMONE RECEPTOR MECHANISMS

1.4.1 Action and Signal Transduction of LHRH

LHRH is synthesized and secreted in a pulsatile manner from the hypothalamus (Melrose et al, 1987; Bourguinon et al, 1987). Gonadal steroids are required for the maturation of LHRH-neurons (Barnea et al,

Table 2: Summary of the effect of hormones on basal and LHRH-induced gonadotropin secretion in primary cultures of rat anterior pituitary cells

Hormone[1]	Basal FSH	Basal LH	LHRH-Induced FSH	LHRH-Induced LH	Reference
Testosterone	↑	--	↑ [↓]	↓ [↓]	Tang and Spies (1975); Drouin and Labrie (1976); Steinberger and Steinberger (1977); Leveque and Grotjan (1982); Kennedy and Chappel (1985); Campen and Vale (1982); [Denef et al (1980)]
Dihydrotestosterone	↑	--	↑ [↓]	↑ [↓]	Drouin and Labrie (1976); Leveque and Grotjan (1982); Massicotte et al (1984a and b) Schwaninger et al (1987); Campen and Vale (1988); [Denef et al (1980)]
17β-Estradiol	↑	↑	↑	↑	Tang and Spies (1975); Debeljuk et al (1978); Steinberger and Chowdhury (1977); Hsueh et al (1979); Lagace et al (1980), Miller and Wu (1981); Massicotte et al (1984a and b) Kotsuji et al (1988); Liu and Jackson (1988)
Progesterone	↑	--	↑	--	Lagace et al (1980); Leveque and Grotjan (1982); Massicotte et al (1984a and b)
Progesterone + 17β-estradiol	↑	↑	↑	↑	Lagace et al (1980); Leveque and Grotjan (1982); Massicotte et al (1984a and b)
Glucocorticoids	↑	↓	↑	↓	Suter and Schwartz (1985); Kamel and Kubajak (1987)
Inhibin	↓	-- [↓]	↓	↓	Lagace et al (1979); Massicotte et al (1984a and b); Fukuda et al (1987); Campen and Vale (1988); [Farnworth et al (1988)]

[1], see text for details of doses, etc.
--, no effect
arrows indicate relative increase/decrease

1988) and for the ongoing regulation of LHRH production and processing in the adult (Karsch et al, 1987; Culler et al, 1988).

At the level of the pituitary, LHRH binds to specific receptors (Wynn et al, 1986) and induces the secretion of LH and FSH (Liu and Jackson, 1984; Jinnah and Conn, 1985). Evidence suggests that this stimulation of gonadotropin release by LHRH also involves the up-regulation of gonadotropin messenger RNA and and therefore gonadotropin synthesis (Stanzec et al, 1986; Kim et al, 1988; Wierman et al, 1989).

Pituitary LHRH receptor number is regulated by gonadal steroids and LHRH itself. Duncan et al (1983) showed that gonadectomy results in increased LHRH receptor number in rat pituitaries and that treatment of such rats with either 17β-estradiol (females) or testosterone (males) restores the pre-gonadectomy number of LHRH receptors. Similar results were obtained in experiments with male rabbits (Limonta et al, 1986). After binding to pituitary cells, LHRH is internalized (Wynn et al, 1986). Prolonged stimulation of gonadotropes with LHRH makes them refractory to further stimulation (Liu and Jackson, 1984; Jinnah and Conn, 1985) indicating LHRH receptor down-regulation, while 30 min pulses of LHRH, which mimic endogenous rhythms, up-regulates or increases LHRH receptor number in the anterior pituitary (Katt et al, 1985).

It was recently concluded that the signal transduction mechanism of LHRH involves the turnover of membrane phospholipids and therefore the production of diacylglycerol and inositol triphosphate, and the subsequent mobilization of calcium and activation of protein kinase C (PKC) (see reviews of LHRH action and receptors: Naor and Childs, 1986; Conn et al, 1987; Clayton, 1989). In studies employing ^{3}H-myo-inositol

or ^{32}P-phosphoinositol, LHRH activation of cultured anterior pituitary cells was shown to result in increased incorporation of the radio-labelled precursors into inositol phosphates, and most importantly, into inositol triphosphate, simultaneous with increased release of LH (Naor et al, 1986; Huckle and Conn, 1987). Naor et al (1986) and Huckle and Conn (1987) also showed that, although phosphoinositide turnover induced by LHRH is independent of calcium, calmodulin or PKC, it is the products of this turnover that elevate intracellular calcium levels (LHRH-induced LH secretion is calcium dependent) and activate PKC, which ultimately results in increased LH release. In a related study, Andrews et al (1986) showed that guanosine triphosphate stimulated an ATP-dependent increase in inositol phosphate production coupled to LH release in permeabilized pituitary cells. This suggests that a guanine nucleotide regulatory (G) protein is functionally linked to the LHRH receptor recognition site.

The elevation of intracellular calcium in gonadotropes is essential to the induction of LH release by LHRH. Chang et al (1986) have shown that LHRH rapidly increases cytoplasmic calcium concentrations in Quin-2 (a compound which fluoresces when exposed to calcium) loaded cultured gonadotropes. In patch clamp studies, Mason and Waring (1986) showed that LHRH opens an inward current, calcium permeable, ion channel in gonadotropes via an internal messenger. Furthermore, studies in which voltage sensitive calcium channels are blocked by drugs such as verapamil and nifedipine demonstrated that the LHRH-induced secretion of LH is inhibited under those conditions (Chang et al, 1986; Chang et al, 1989). In these experiments LH release is not totally inhibited and it was suggested that calcium release from intracellular stores initiates

LH release, while influx of calcium from external sources maintains the response. In all of the above-mentioned studies, the calcium ionophore A23187 mimicked the effects of LHRH on LH release.

The response of pituitary cells to LHRH also involves the activation of protein kinase C (PKC) by diacylglycerol (from phosphoinositide turnover) and calcium (Naor and Childs, 1986; Conn et al, 1987; Clayton, 1989). Phorbol esters and diacylglycerol, which activate PKC, (Nishizuka, 1986) enhance the secretion of LH in cultured anterior pituitary cells (Negro-Vilar and Lapetina, 1985; Liu and Jackson, 1987; McArdle et al, 1988). It was suggested that activation of PKC by LHRH is involved in the longer-term maintainance of the LH response to LHRH since PKC activation not only increases glycosylation of pituitary LH (Liu and Jackson, 1987), but the immediate effects of LHRH are more closely mimicked by the calcium ionophore A23187 or high concentrations of potassium (a secretagogue) (Naor and Childs, 1986; Conn et al, 1987; Clayton, 1989). PKC activation by LHRH might also play a role in LHRH receptor regulation since refractoriness of LH release to LHRH is calcium independent and involves some other post-receptor mechanism (Jinnah and Conn, 1985). Inhibition of glycosylation with tunicamycin interferes with the restoration of pituitary responsiveness to LHRH (Jinnah and Conn, 1985).

1.4.2 Mechanism of Action of Steroids

The majority of steroid actions are mediated through nuclear receptors which regulate gene expression. Receptors for gonadal steroids and glucocorticoids are localized in the nucleus of target cells (O'Malley and Schrader, 1976; Clarke, 1984; King and Greene, 1984;

Welshons et al, 1985) and further, are associated with non-histone proteins of the nuclear matrix (Barrack, 1984; Barrack, 1987; Spelsberg et al, 1987). Activation of the steroid-receptor complex, at least in the chick oviduct progesterone receptor, appears to involve receptor subunit phosphorylation (Garcia et al, 1987). Steroids regulate gene expression in many of the target tissue systems studied. Progesterone and estrogen stimulate the synthesis of vitellogenin and albumin in the chick liver and oviduct systems (O'Malley and Schrader, 1976; Chambon et al, 1984). A recent study by Shupnik et al (1988) showed that 17β-estradiol suppresses the transcription of gonadotropin genes in the rat. Genes regulated by steroids are ubiquitous, with many occurring in the brain, hypothalamus and pituitary (see Beato et al, 1987; Harlan, 1988; Spelsberg et al, 1989 for complete reviews).

New evidence suggests that steroids also interact with and/or bind to plasma membranes. Towle and Sze (1983) first showed that gonadal steroids bind to synaptic plasma membranes with considerable affinity (dissociation constants in the nanomolar range). Progesterone can increase the membrane potential and the release of acetylcholine in frog neuromuscular junctions (Meiri, 1986). A specific binding site for 17β-estradiol has been found on the membranes of rat anterior pituitary cells (Bression et al, 1986) and both 17β-estradiol and testosterone induce the formation of exo-endocytotic pits in hypothalamic neurons (Garcia-Segura et al, 1987). Endogenous steroids also modulate the activity of gamma amino-butyric acid (GABA) receptor-benzodiazapine receptor complexes in the central nervous system. Pregnenolone sulphate and A-ring reduced metabolites of pregnenolone antagonize the activity of GABA complexes and have thus been dubbed "neurosteroids" (Lambert et

al, 1987; Majewska and Schwartz, 1987). The analgesic properties of 3α-hydroxy-5α-pregnan-20-one involves its interaction with the GABA complex: its action is blocked by GABA antagonists, reduced by opiate and benzodiazepine antagonists and enhanced by calcium channel antagonists (Kavaliers and Wiebe, 1987).

Steroids may also interact with the signal transduction mechanism of protein hormones such as LHRH. Several studies have indicated that 17β-estradiol enhances LHRH-induced secretion of LH and FSH by interaction with the calcium and/or diacylglycerol (PKC) pathways after cell activation by LHRH (Liu and Jackson, 1987; Liu and Jackson, 1988).

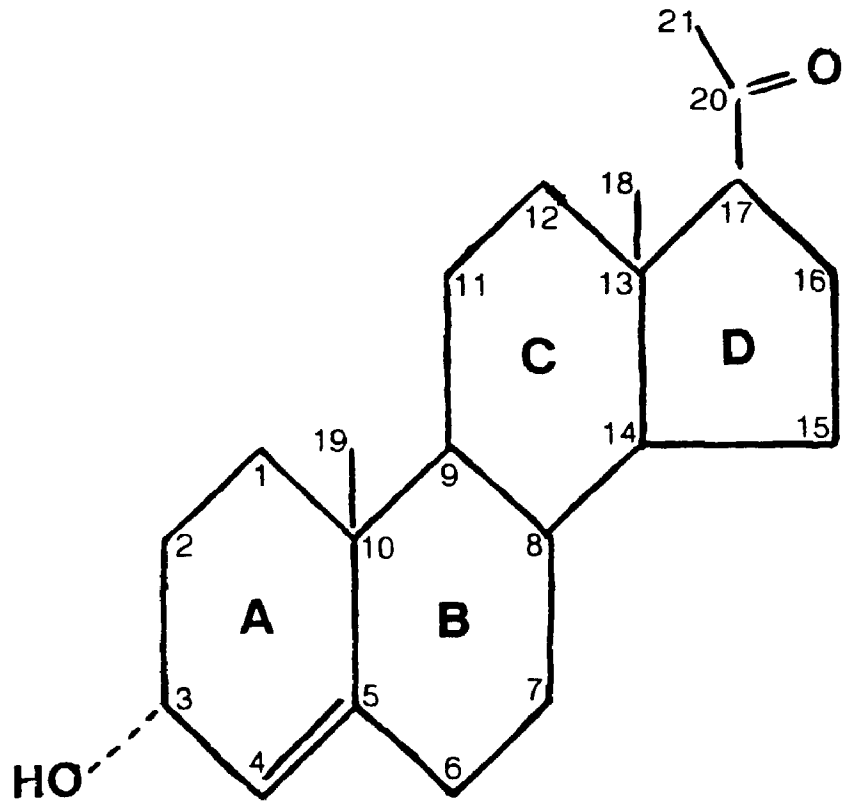
1.5 A NOVEL GONADAL STEROID, 3α-HYDROXY-4-PREGNEN-20-ONE

1.5.1 The Discovery of 3α-hydroxy-4-pregnen-20-one (3HP)

In the late 1970's and early 1980's Wiebe and coworkers studied the ability of isolated rat Sertoli cells to synthesize and metabolize steroids. These studies showed that isolated Sertoli cells from rats could metabolize androgens (Welsh and Wiebe, 1978), metabolize progesterone and pregnenolone (Wiebe, 1978b; Wiebe et al, 1980) and synthesize C_{21} steroids de novo from acetate (Wiebe and Tilbe, 1979). Sertoli cell steroidogenesis is stimulated by FSH (Welsh and Wiebe, 1976; Tilbe and Wiebe, 1981). In the above studies one of the more abundant metabolites was dubbed "#4" for the elusive nature of its identity. Careful work employing gas chromatography, high performance liquid chromatography, thin layer chromatography, mass spectrometry and chemical manipulation finally identified this compound as 3α-hydroxy-4-pregnen-20-one (3HP; Figure 3) (Wiebe, 1982) which accounts for 7% of the products of Sertoli cell progesterone metabolism (Wiebe et al,

Figure 3: Chemical structure of the allylic gonadal steroid, 3α-hydroxy-4-pregnen-20-one (3HP). 3HP is a C_{21} steroid containing the allyl group in ring A. 3HP differs from progesterone only by the substituent at position 3 in the A ring: progesterone has a ketone group here while 3HP has an hydroxyl group in the α-orientation. Stereochemically, 3HP has rings A and B in a single plane with the 3α-hydroxyl group sticking out from the plane of rings A and B.

3α-HYDROXY-4-PREGNEN-20-ONE (3HP)



1980). 3HP does not appear to be produced by Leydig cells. 3HP has since been shown to be a product of domestic hen thecal cells (Marrone et al, 1985) and cultured rat granulosa cells (Wiebe pers. comm., 1988). Although 3HP is a labile molecule, a synthetic technique has been developed and milligram quantities of the steroid can be synthesized (Wiebe et al, 1985). Wiebe (1985) provides a good review of Sertoli cell steroidogenesis-particularly the production of 3HP.

1.5.1 Properties of 3HP

Sertoli cell steroid metabolism is stimulated by FSH (Tilbe and Wiebe, 1981). Careful studies showed that the production of 3HP is age-dependent with peak amounts produced by Sertoli cells from 10-17 day old rats and steady smaller amounts produced well into adulthood (Wiebe, 1982). This relatively high level of 3HP coincides with the time when FSH levels are low in the male rat (Dohler and Wuttke, 1975), FSH binding in Sertoli cells is maximal (Salhanick and Wiebe, 1980) and meiosis is about to begin (Wiebe, 1982). Wiebe et al (1988) review the Sertoli cell-specific production of 3HP.

Recent work (Wiebe and Kavaiers, 1988) has demonstrated that 3HP has an analgesic effect when injected intracerebroventricularly. 3HP analgesia appears to involve interaction with the GABA receptor complex in the central nervous system since GABA, benzodiazepine and opioid antagonists block its effect while the dihydropyridine-sensitive calcium channel antagonists enhanced the analgesic effect of 3HP (Wiebe and Kavaliers, 1988).

1.6 OBJECTIVES AND APPROACHES

The main objective of this study was to determine whether 3HP plays a role in the regulation of gonadotropin secretion in the male rat. Many gonadal steroids play a part in the regulation of LH and FSH secretion (Table 1) and 3HP too, may play a role. In particular, regulation of FSH secretion in the male rat is not fully understood and it seems likely that 3HP might play a role since 3HP is produced in considerable quantities by rat Sertoli cells (and not Leydig cells) in an age- and FSH-dependent fashion. The unique structure of 3HP may also confer unique properties upon it.

This problem was approached using the prepubertal male rat model (both intact and gonadectomized) treated with 3HP and the circulating levels of gonadotropins were examined using radioimmunoassay (RIA). Rat anterior pituitary cells in culture were also employed to further examine the effect of 3HP on gonadotropin secretion.

These models were chosen since 3HP was first isolated from rat Sertoli cells and there is a wealth of information regarding the role of gonadal steroids in the regulation of pituitary gonadotropin secretion in the rat.

Specifically, an attempt was made to answer the following questions:

(1) What effect does 3HP have on gonadotropin secretion (particularly FSH secretion) in the male rat?

(2) Is this effect dose-dependent and therefore of physiological significance?

(3) Is this effect age-dependent?

(4) Is this effect distinct from that of functionally-related

steroids?

(5) Does 3HP have a similar effect on cultured anterior pituitary cells?

(6) Is the in vitro 3HP effect dose-dependent?

(7) Is this effect unique to 3HP or is it similar to that of funtionally or structurally related steroids?

(8) By what mechanism does 3HP act? Does it interact with the signal transduction pathway of LHRH?

MATERIALS AND METHODS

2.1 CHEMICALS

2.1.1 Steroids

The following steroids were purchased from Sigma Chemical Company (St. Louis, Mo.): 17β-hydroxy-4-androsten-3-one (testosterone), 17β-hydroxy-5α-androstan-3-one (dihydrotestosterone), 1,3,5(10)-estratriene-3,17β-diol (17β-estradiol; estrogen), 4-pregnene-3,20-dione (progesterone), 17α-hydroxy-4-pregnene-3,20-dione (17α-hydroxyprogesterone), 20α-hydroxy-4-pregnen-3-one (20α-dihydroprogesterone), 20β-hydroxy-4-pregnen-3-one (20β-dihydroprogesterone), 5α-pregnane-3,20-dione (5α-dihydroprogesterone), 5β-pregnane-3,20-dione (5β-dihydroprogesterone), 3β-hydroxy-5α-pregnan-20-one and 3β-hydroxy-5β-pregnan-20-one. 3α-Hydroxy-5α-pregnan-20-one and 3α-hydroxy-5β-pregnan-20-one were purchased from Steraloids (Wilton, N.H.). 3α-Hydroxy-4-androsten-17-one, 3α-hydroxy-4-pregnen-20-one 3α-dihydroprogesterone; 3HP), 3β-hydroxy-4-pregnen-20-one, 3α-hydroxy-4-pregnen-20-one-3-acetyl, 3α,20α-dihydroxy-4-pregnene and 3α,20β-dihydroxy-4-pregnene were synthesized in our laboratory as described (Wiebe et al, 1985; Wiebe et al, 1986).

2.1.2 Drugs and Hormones

Synthetic luteinizing hormone-releasing hormone (pGlu-his-trp-ser-tyr-gly-leu-arg-pro-gly-NH_2, acetate salt; LHRH), verapamil (α-[3-[[2-(3,4-dimethoxyphenyl)ethyl]-methlyamino]propyl]-3,4-dimethoxy-α-(1-methylethyl)benzeneacetonitrile) and nifedipine (1,4-dihydro-2,6-dimethyl-4-(2-nitro-phenyl)-3,5-pyridinedicarboxylic acid dimethyl ester) were

purchased from Sigma Chemical Company (St. Louis, Mo.). The calcium ionophore A23187 (calimycin) was supplied by Boehringer-Mannheim (West Germany). The protein kinase C inhibitor H-7 (1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride) and protein kinase C acitivator SC-9 (N-(phenylhexyl)-5-chloro-1-naphthalenesulfonamide) were purchased from Seikagaku America (St. Petersburg, Fl.). I am indebted to Dr. Martin Kavaliers for the generous gift of the A23187, verapamil, nifedipine, H-7 and SC-9.

2.1.3 General

Medium 199 (M199), collagenase Type I, trypsin, penicillin-G and Sephadex G-75 were from Sigma Chemical Company (St. Louis, Mo.). Streptomycin-sulphate, lactoperoxidase and bovine serum albumin were from Boehringer-Mannheim (West Germany).

The rest of the chemicals used in these experiments were of reagent grade and were purchased from either Sigma Chemical Company (St. Louis, Mo.) or BDH (Toronto, Ont.).

2.2 ANIMALS

2.2.1 Source and Housing

All rats used in this study were of the Sprague-Dawley strain and were either purchased from Charles River Breeders (St. Constant, Que.) or were bred in our own facilities from Charles River stock. Rats were housed at no more than 3 adults or 6 juveniles per cage and kept on a 14-hr light, 10-hr dark photoperiod (lights on at 0600 h, off at 2000 h) at 24°C. Food (Purina or Agwayer rat chow) and water were supplied ad libitum. Newborn rats were kept with mothers until weaning at 21 days of

age. All mature females used in this study were random-cycling.

2.2.2 Surgical Procedures

All gonadectomies were performed under aseptic conditions using light ether anesthesia. For pubertal male rats a small (1 cm) incision was made in the mid-ventral region of the scrotum; in prepubertal male rats an incision was made mid-ventrally just anterior to the penis. The testis was externalized and the epididymis was carefully trimmed away from the lateral surface of the testis. A suture (4-0 silk) was securely tied at the anterior end of the testis to ligate the epididymis and blood vessels before removal of the testis itself. The remaining tissue was then replaced into the scrotal cavity and the procedure repeated for the other testis. The incision was closed with 9 mm wound clips.

Female rats were ovariectomized via a small dorsal incision just anterior to the hip and lateral to the midline. The ovary was externalized, a hemostat applied to the oviduct and ovarian artery and the ovary and fatty tissue carefully trimmed away. The hemostat was left in place for about 30 sec and then removed. The incision was closed with a 9 mm wound clip and the procedure repeated for the other side.

All animals were allowed to recover under a heat lamp for about 2 hr post-surgery and then returned to the animal quarters. A further 2 days rest was allowed before animals were used in any experiment.

2.3 IN VIVO EXPERIMENTS

2.3.1 Preparation of Steroids

All steroids were initially dissolved in glass-distilled ethanol (mg/ml), the required amount added to a sterile serum vial and then

dried down under a stream of nitrogen. Glass-distilled ethanol (to make up 10% of the total volume of solution) was added to the vial to dissolve the steroid, followed by sterile saline-Tween 80 (0.9% sodium chloride, 0.1% Tween 80 in distilled water) to total volume. For high doses of steroids, glass beads were added to keep the suspension uniform. Solutions were made fresh on the first day of treatment, stored at 4°C and warmed to room temperature before use.

2.3.2 Treatment Regimens

Doses were given on a per 100 g body weight (BW) basis, where body weight was considered to be the calculated average body weight of animals on the middle day of the experiment. For example, if the average weight of rats on day 1 of the experiment was 60 g and the experiment was to continue for 5 days, then the calculated average weight on day 3 was 70 g, assuming a 5 g/day weight gain in the prepubertal rat. Steroids were administered subcutaneously (s.c.) in 100 µl of vehicle using a 23G needle and a 1 ml syringe. Intact animals were injected in the morning between 1000 and 1200 h for days 1-4 of the experiment and were sacrificed 24 hr after the last injection. Treaments on gonadectomized animals were begun on the 3rd day after surgery (day 1), when the post-castration rise in serum gonadotropins is complete in the juvenile animal, and continued every morning for a total of 4 treatments and rats were sacrificed on the morning of day 5.

2.3.3 Serum Sampling and Storage

On day 5 of the experiment, animals were sacrificed by decapitation with a small mammmal guillotine (Harvard Bioscience) after being

rendered unconscious in a carbon dioxide (CO_2) chamber. Trunk blood was collected and allowed to clot for 4 hr at room temperature. Serum was separated by centrifugation at 1500 x g for 15 min in a refrigerated Beckman TJ-6 centrifuge and removed with a clean pasteur pipette. Serum was stored at -20°C until assayed for gonadotropins.

Figure 4 shows an overall scheme of the protocol employed for the in vivo studies of gonadotropin secretion in the rat.

2.4 IN VITRO EXPERIMENTS

The procedures outlined here for the isolation, maintenance and experimental manipulation of anterior pituitary cells in culture are a modification of the technique of Kennedy and Chappel (1985). Modifications were based on the literature outlined in Table 2.

2.4.1 Prepartion of Cell Cultures

Random-cycling adult female rats were used unless otherwise indicated in the Results. Rats were rendered unconscious in a CO_2 chamber and swiftly decapitated. The rest of the procedure was carried out under sterile conditions in a tissue culture hood using Falcon sterile tissue culture ware (Becton-Dickinson). Skin was cut away from the dorsal surface of the cranium to expose the underlying muscle and bone. Scissors were used to open the cranium along the suture between the parietal and squamosal bones and the roof of the skull lifted off. The brain was carefully reflected with a smooth scoop to expose the underlying pituitary gland. The enclosing membrane was removed with fine forceps and then the pituitary was removed and placed on ice in sterile Kreb's Ringer Phosphate (KRP; 118 mM sodium chloride, 4.7 mM potassium

Figure 4: Schematic representation of the protocol followed for all *in vivo* experiments. See sections 2.2.2 and 2.3 for further details. Note that in experiments conducted on intact animals the actual regimen began at part 2:**TREATMENT**.

IN VIVO PROTOCOL

GONADECTOMY light ether anesthesia

2 days rest

TREATMENT 4 consecutive days

vehicle: saline with 10% glass-distilled

ethanol and 0.1% Tween 80

SACRIFICE day 5

decapitation

trunk blood collected and allowed to clot 4hrs

serum stored at $-20^{\circ}C$

RADIOIMMUNOASSAY

determine serum FSH and LH levels on duplicate samples

chloride, 5.5 mM glucose, 116 μM magnesium sulfate, 190 μM calcium chloride, 710 μM sodium bicarbonate and 4 mM glutamine made up in 10 mM Sorenson's phosphate buffer [8.4 mM dibasic sodium phosphate and 1.6 mM monobasic potassium phosphate], pH 7.4) supplemented with penicillin-G (100 U/ml) and streptomycin-sulfate (100 μg/ml). Pituitaries were washed 6x with sterile, supplemented KRP and minced with razor blades before being transferred to a 15 ml conical tube containing collagenase (0.25%) in unsupplemented KRP. Cells were dispersed at 37°C in a shaking water bath (American Optical) for 45 min, then pelleted in a clinical centrifuge (IEC) at 80 x g for 10 min. The supernatant was removed and cells brought up in fresh culture medium (M199 with Hank's salts supplemented with 4 mM glutamine, 10% charcoal-treated fetal calf serum 100 U/ml penicillin-G and 10 μg/ml streptomycin-sulfate, pH 7.4). Cells were disaggregated by repeated aspiration (approximately 200 x) in a Pasteur pipette and a small (100 μl) aliquot taken to determine cell number and viability. Cells were diluted in fresh culture medium to final culture concentration (3-8 x 10^5 cells/ well) in a stir-flask (Wheaton) to maintain a uniform density and plated at either 2 ml per well in a 6-well plate or 1 ml per well in a 24-well plate. Cells were then incubated at 37°C and 95%-air, 5%-CO_2 in a Narco 5100 incubator for 72 hr before use.

2.4.2 Treatment of Calf Serum

Fetal calf serum was treated with 1% charcoal and 0.1% dextran for 4 hr at room temperature to remove endogenous steroids. Serum was centrifuged at 20,000 x g in a refrigerated Sorval RC2B centrifuge for 40 min to remove charcoal. The supernatant was decanted into the M199

and the total medium sterile-filtered before use.

2.4.3 Determination of Cell Number and Viability

Trypan blue dye solution (10 µl; 0.4% trypan blue, 137 mM sodium chloride and 4.4 mM monobasic potassium phosphate, pH 7.2) was added to the 100 µl aliquot of concentrated cell suspension and the mixture allowed to stand for 4 min. Cells were loaded into a hemocytometer and viable and non-viable cells were counted in the 10 large squares. Cell number was determined as the total number of viable cells/ml of suspension. Viability was determined as the percent of total cells which remained unstained by the trypan blue. Only anterior pituitary cell preparations whose viability was greater than 90% were used in this study.

2.4.4 Protein Determination

From 3-5 samples, equivalent to 2 wells of culture supension, were taken from the diluted culture suspension throughout the seeding procedure. Each sample was centrifuged at 80 x g in a clinical centrifuge at room temperature, washed once at room temperature in phosphate buffered saline (PBS; 2.7 mM potassium chloride, 1.5 mM monobasic potassium phosphate, 137 mM sodium choride and 8.1 mM dibasic sodium phosphate pH 7.4) and suspended in 500 µl PBS. Samples were sonicated for 10 sec at setting 2 (Insonator, Ultrasonic Systems, Inc.) and stored at -20°C until assayed. Protein was determined by the method of Bradford (1976). Briefly, 3 ml of Bradford reagent (0.67% Coomassie brilliant blue dissolved in a solution of 33% of 95% ethanol and 67% of 85% phosphoric acid diluted 6.7 times with distilled water for use) was

added to 200 µl of cell sample and allowed to stand for 10 min. Samples were then read at 595 nm on a Unicam SP1800 spectrophotometer (Canlab) and the protein value determined from a standard curve produced with bovine serum albumin (BSA).

2.4.5 Steroid Treatments

On the day of treatment, culture medium was removed and cells washed with sterile, supplemented KRP. Experimental medium (M199 with Hank's salts and 4 mM glutamine, pH 7.4) was added to all wells followed by the various treatments. Steroids were diluted in glass-distilled ethanol and added in a 20 µl (2 ml well) or 10 µl (1 ml well) volume to give the final culture concentration as indicated. LHRH was dissolved in 0.01 M acetic acid and sterile-filtered before being added in a 20 µl (2 ml well) or 10 µl (1 ml well) volume to give a final culture concentration of 10 nM (except where noted). Controls received the appropriate amount of ethanol and/or 0.01 M acetic acid only. Cells were returned to the incubator for a further 4 or 24 hr before sampling.

2.4.6 Drug Treatments

Several drugs were employed in an attempt to discern the mechanism of action of the inhibition of basal and LHRH-induced FSH secretion by 3HP in anterior pituitary cells in culture. The calcium channel blockers verapamil and nifedipine and the calcium ionophore A23187 were dissolved in glass-distilled ethanol (500 µl) and added to 50 ml of experimental medium to give a final concentration of 100 µM. 3HP and LHRH doses were given as in section 2.4.5 (above).

The protein kinase C inhibitor, H-7, was dissolved in PBS (4 mg/ml)

and 216.5 µl added to 50 ml of experimental medium to give a final concentration of 50 µM. The protein kinase C activator SC-9 was dissolved in glass-distilled ethanol (4 mg/ml) and 201 µl added to 50 ml of experimental medium to give a final concentration of 100 µM. 3HP and LHRH treatments were given as above (Section 2.4.5).

2.4.7 Media and Cell Sampling

After 4 or 24 hr incubation, medium from each well was collected with a clean pasteur pipette and stored at -20°C in disposable glass tubes (12 x 75 mm) until assayed for gonadotropin content. In cases where cells were also collected, the following procedure was used: medium was removed and 500 µl of 0.1% trypsin (Sigma, Type II) in PBS was added to each well and the cells allowed to incubate at 37°C for 5 min. Cells were then gently scraped from the surface of each dish, the dishes were rinsed with 500 µl of PBS and the resulting sample centrifuged at 800 x g (Beckman TJ-6) for 5 min. The supernatant was discarded, 1 ml of fresh PBS added to the cell pellet and each sample sonicated for 5 sec at setting #2 on an Insonator (Ultrasonic Systems, Inc.). Samples were again centrifuged as above and the supernatant retained for assay of cell content of gonadotropins. These samples were stored at -20°C in polypropylene culture tubes (12 x 75 mm).

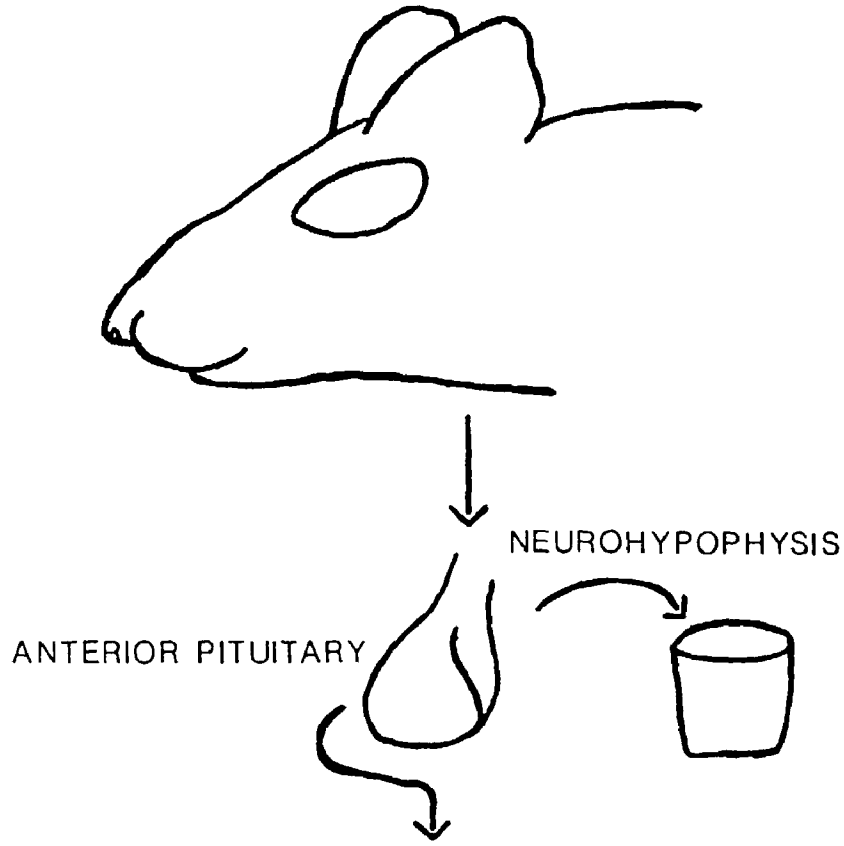
Figure 5 shows a generalized scheme of the procedures used in the *in vitro* studies.

2.5 RADIOIMMUNOASSAY OF GONADOTROPINS

2.5.1 Preparation of Sephadex Columns

Columns for the separation of iodinated hormones from the reaction

Figure 5: Schematic representation of the protocol followed for all *in vitro* experiments. See section 2.4 for full details on procedures used. Note that some incubations were carried out for only 4 hr while the majority were allowed to continue for 24 hr.



M199 (10% DCC-FCS; 4mM glutamine)
72 hrs at 37°C; 95%-air, 5%-CO2

M199 (4mM glutamine only)
Steroids added in 20 ul glass-distilled EtOH
LHRH added in 20 ul 0.01M acetic acid

Incubate 24 hrs. as above

Collect medium and cells for analysis of gonadotropins by RIA

buffers were prepared using 10 ml disposable glass pipettes. Sephadex G-75 gel was swollen overnight at 4°C in distilled water before pouring. PBS was added to keep the gel moist and the system allowed to flow until a 6 ml packed bed of gel was established. Five percent BSA in PBS was run into the gel to saturate any sites where large proteins may adsorb. Two bed volumes of PBS (ie. 12 ml) were used to ensure that all the non-adsorbed BSA was flushed from the gel. The eluent was allowed to drain just to the surface of the gel to prepare the column for use.

2.5.2 Radioiodination

The National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) provided the hormones for iodination. Preparations used were rat follicle-stimulating hormone (rFSH-I-5, rFSH-I-6 and rFSH-I-7) and rat luteinizing hormone (rLH-I-5, rLH-I-6 and rLH-I-7). These preparations were provided as lyophilized powders and were rehydrated with PBS to a working concentration of 100 µg/ml and stored at -70°C in 25 µl aliquots in disposable 1.5 ml microcentrifuge tubes. Aliquots were thawed on ice just prior to iodination. Iodinations were carried out in a lead-shielded fume hood using the lactoperoxidase method of Mougdal et al (1978). Iodine-125 (^{125}I, 2.5 millicuries; ICN Biochemicals) were added to 1.25 µg of hormone preparation and mixed with slight agitation (in the microcentrifuge tube). Lactoperoxidase (10 µl; 100 µg/ml in 0.4 M sodium acetate, pH 5.5) was then added with slight agitation. Dilute hydrogen peroxide (5 µl;0.001%) was then added and the reaction vessel agitated for 30 sec and allowed to sit for 60 sec. The hydrogen peroxide step was repeated once and the reaction terminated with 100 µl of 5% BSA in PBS. The reaction mixture was then carefully layered onto the gel bed

of a Sephadex G-75 column. BSA in PBS (0.1%; 100 µl) was used to rinse the microcentrifuge tube and the rinse was layered onto the gel. The clamp was opened to allow the sample to flow into the gel and then closed. The reservoir segment of the column was attached and carefully filled with the eluent (0.1% BSA in PBS). The clamp was opened and 10-drop fractions of eluate collected in 10 disposable glass culture tubes (12 x 75 mm). Each fraction was sampled (5 µl) and counted in a Beckman Gamma 4000 gamma spectrometer equipped with a DP5000 microprocessor. Fractions containing the first peak of radioactivity were pooled (usually numbers 3, 4 or 5) as they contained the iodinated hormone (Sephadex G-75 columns exclude large proteins and retain small molecules such as ions in the buffer and unreacted ^{125}I). The pooled labelled hormone was aliquoted (100 µl) and stored at -20°C in disposable glass tubes (12 x 75 mm) until use. All iodinated hormones were discarded 45 days after iodination (they begin to lose their ability to bind to specific antibodies after this time).

2.5.3 Storage and Preparation of Standards and Primary Antibodies

The hormone standards were provided by the NIDDK in lyophylized form and were rehydrated with distilled water and stored at -70°C in the following aliquots: 100 µl rLH RP-1 or rFSH RP-1 (100 µg/ml), 25 µl rLH RP-2 (5 µg/ml) and 25 µl rFSH RP-2 (10 µg/ml). On the day of the assay, these standards were serially diluted in assay buffer (see below) to known amounts per tube on the day of the assay. The primary antibodies were provided in a 1 ml volume at a dilution of 1:12.5 for NIDDK-anti-rFSH-S-11 and 1:18.75 for NIDDK-anti-rLHh-S-8 and were further diluted to 1:62.5 and 1:75, respectively, with antibody diluent (see below) and

stored at -70oC in 25 ul aliquots until the day of the assay. Primary antibodies had been raised in rabbits against the rat hormones.

2.5.4 Radioimmunoassay of Serum and Media Samples

Serum, media and cell samples were assayed for gonadotropins by RIA as follows. Duplicate samples of serum (200 µl) or medium or cell homogenate (150 µl) were added to 12 x 75 mm disposable glass tubes and the volume brought up to 500 µl with assay buffer (10 mM diabasic sodium phosphate, 10 mM monobasic sodium phosphate, 148 mM sodium chloride with 1% bovine serum albumin and 0.01% thimerosal as a preservative). Primary antibodies were diluted with antibody diluent (10 mM dibasic sodium phosphate, 10 mM monobasic sodium phosphate, 50 mM ethylenediaminetetraacetic acid [EDTA] with 2% normal rabbit serum) to a factor of 1:31,250 (anti-rFSH) or 1:45,000 (anti-rLH) and added in a 200 µl volume to the assay tubes. Iodinated FSH (20,000 cpm/tube) or LH (40,000 cpm/tube), depending on the assay being performed, was then added to all tubes in a 100 µl volume of label diluent (10 mM dibasic sodium phosphate, 10 mM monobasic sodium phosphate, 148 mM sodium chloride with 0.1% bovine serum albumin and 0.01% thimerosal as a preservative). The final dilution of the antibodies was therefore 1:125,000 for anti-rFSH and 1:180,000 for anti-rLH. Total binding of label by antibody was determined in triplicate tubes containing only assay buffer and primary antibody and non-specific binding (NSB) was determined in tubes containing only assay buffer and antibody diluent. Standards were also assayed in triplicate. All tubes were allowed to incubate at room temperature for 24 hr before the addition of the secondary antibody. A polyclonal serum goat anti-rabbit IgG (Daymar

Laboratories Inc., Toronto, Ont.) was diluted with assay buffer and added in a 100 µl volume to all tubes to precipitate the hormone-antibody complexes. After 24 hr, tubes were centrifuged at 4°C and 1500 x g for 15 min (Beckman TJ-6), the supernatant aspirated and the bound hormone counted for 1 min in a Beckman Gamma 4000 gamma radiation counter. Total specific radioactivity bound (B_o) was calculated as the total bound radioactivity in tubes with no exogenous hormone added minus the radioactivity bound in the absence of primary antibody (NSB). Specific bound radioactivity (B) for all standards and samples was calculated as the radioactivity bound per tube minus the NSB value. The ratio of $B:B_o$ was plotted against the amount of hormone for all standards and this curve was used to calculate the amount of hormone present in each samples. All calculations were made with the aid of a computer program and all procedures are continuously validated by our lab.

2.5.5 Assay Reliability and Sensitivity

Reliability between RIA's was determined by inclusion of a sample of pooled male rat serum in every assay. The coefficient of variation for these samples was 16.75% (FSH) and 16.38% (LH) which is considered acceptable. Intra-assay variation, determined between replicate samples, was always less than 15%.

The RP-1 hormone standards used in the earlier work had the working range of 1.5-100 ng for LH and 10-1000 ng for FSH. The RP-2 standards were much more sensitive than the RP-1 standards; rFSH RP-2 and rLH RP-2 are 45x and 61x more potent, respectively, than their RP-1 counterparts. Therefore, a much lower working range of 0.05-10 ng for LH and 0.1-25 ng

for FSH was used with the RP-2 standards. Data are presented in terms of RP-1 for the *in vivo* work and RP-2 for the *in vitro* work. Some of the culture data were originally obtained using RP-1 standards but were later converted to RP-2 equivalents (using NIDDK conversion factors) to facilitate comparisons between experiments.

2.6 STATISTICAL ANALYSIS

In all experiments a minimum of three replicates was used for every treatment group. For most of the studies, a simple comparison of the treatment means was carried out using the two-tailed Student's t-test unless otherwise indicated. The 3HP dose-response data were analysed by ANOVA and curves fitted for *in vitro* results using regression analysis. Experiments on the interaction of 3HP with the signal transduction pathway of LHRH were subjected to factorial analysis to confirm main effects and determine interactions among treatments.

RESULTS

3.1 FSH-SUPPRESSING EFFECT OF 3HP IN VIVO

3.1.1 Prepubertal Intact Male Rats

Figure 6 shows that 3α-hydroxy-4-pregnen-20-one (3HP) administered in various doses to intact 26 day old male rats for a period of 5 days resulted in a significant selective suppression of serum FSH levels at the three highest doses (20, 40 and 80 μg/100 g BW; $p<0.001$, $p<0.01$ and $p<0.001$, respectively). Circulating FSH was decreased to approximately 75% of the control level by all three doses. Serum LH levels were significantly increased by the 10 μg/100 g BW 3HP dose ($p<0.05$) but this is the only case where this result was seen. These results were pooled from several experiments conducted under identical conditions.

In an effort to determine the optimum number of injections of 3HP required to observe its FSH-suppressing effect in intact prepubertal male rats, groups of animals were treated with 80 μg/100 g BW 3HP for 1-5 days and sacrificed either 8 or 24 hr after the first injection or 24 hr after each succeeding injection. Table 3 shows that, at all sampling times, FSH was specifically inhibited by 3HP ($p<0.01$). As early as 8 hr following a single injection, serum FSH was suppressed by about 50% ($p<0.001$). On the other hand, no significant effect on serum LH was observed. Based on this information, it was decided that all subsequent experiments using the intact prepubertal male rat model would be conducted using a single treatment with the steroid to be tested.

Sensitivity of the hypothalamic-pituitary-gonadal axis to 3HP is already present at a very young age. Various doses of 3HP were administered in a single injection to 10 and 20 day old male rats

Figure 6: Effect of 3α-hydroxy-4-pregnen-20-one (3HP) on serum FSH () and LH () levels in 26 day old intact male rats. Animals were given 4 daily s.c. injections of 3HP (0, 5, 10, 20, 40 or 80 µg/100 g BW) in saline vehicle and sacrificed on the fifth day (24 hr after the last injection). Measurements of serum FSH and LH by RIA were made on duplicate 200 µl samples. Bars represent the mean±SEM of n=5-14 animals (the results of several experiments conducted under identical conditions were pooled for this figure) and results are expressed as ng RP-1/ml of serum.

*$p<0.05$, **$p<0.01$ and ***$p<0.001$ compared to vehicle-treated control

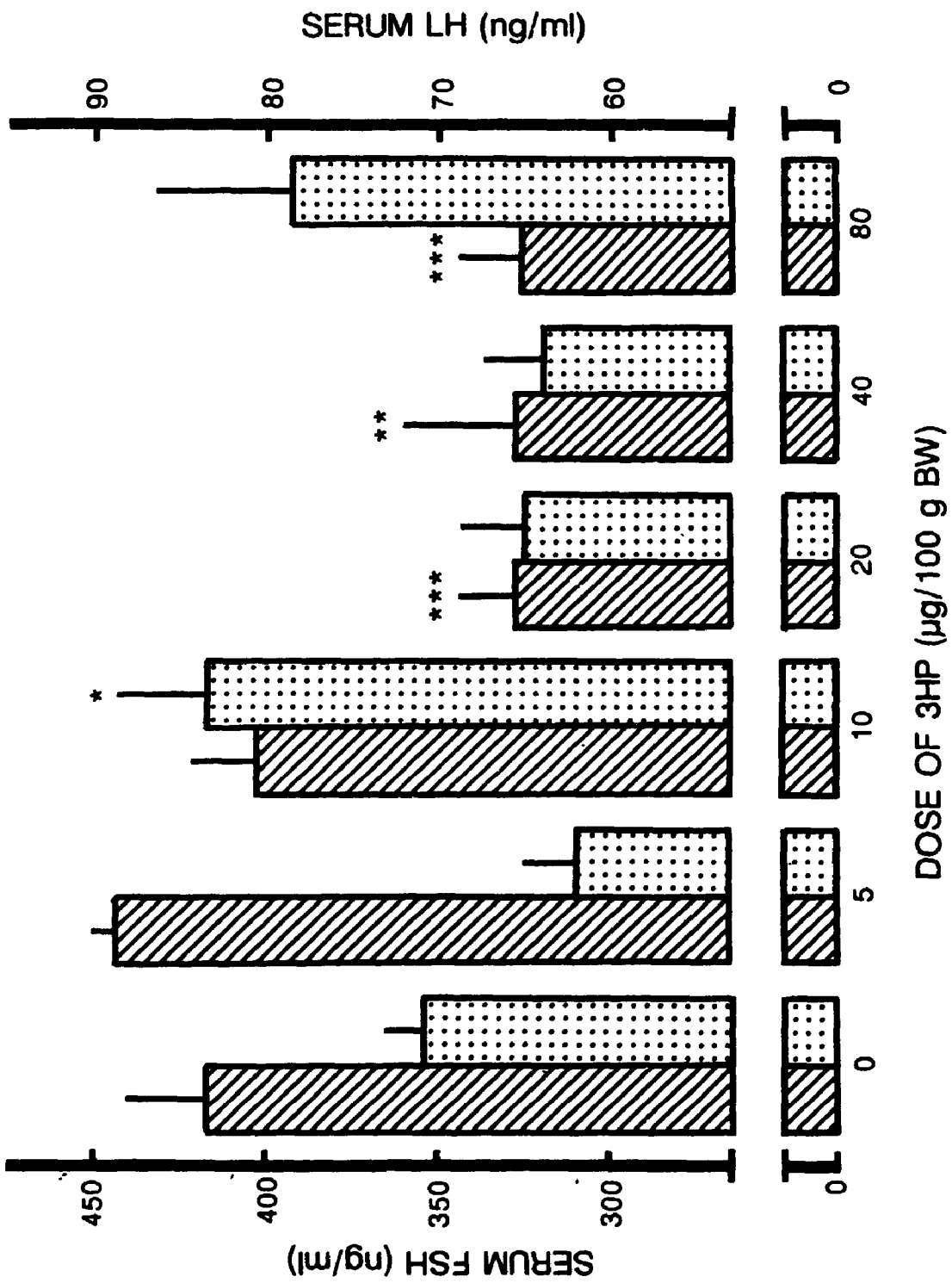

SERUM LH (ng/ml)
90
80
70
60
0
***
**
***
*
0
5
10
20
40
80
DOSE OF 3HP (µg/100 g BW)
450
400
350
300
0
SERUM FSH (ng/ml)

Table 3: Effect of the number of administered doses of 3α-hydroxy-4-pregnen-20-one (3HP) on gonadotropin secretion in intact, juvenile male rats.

No. of injections	Serum FSH (ng/ml)	Serum LH (ng/ml)
Control	443.2±24.6	74.0±16.6
1 (8 hr)	222.7±30.9[b]	68.1± 2.6
1 (24 hr)	268.0±22.6[b]	80.4±10.9
2	349.7± 7.5[a]	69.6± 8.0
3	291.0±15.1[b]	64.2±12.7
4	330.3±16.1[a]	58.7±28.1
5	253.0±26.9[a]	33.4±13.2

Intact 26 day old male rats were treated as described (Section 2.3) with 1-5 injections (s.c.) of 80 ug/100 g BW 3HP on a daily basis and sacrificed 24 hr after the last injection (except where indicated). Results shown are the mean±SEM of n=4-6 replicates and are expressed as ng RP-1/ml of serum.

[a]$p<0.01$ and [b]$p<0.001$ compared to control.

(Figure 7) and all doses (20, 40 and 80 μg/100 g BW) decreased serum FSH levels in the 20 day old rat (0.001). Only the 20 and 80 μg/100 g BW doses of 3HP suppressed serum FSH in the 10 day old rat. Serum LH levels were not affected by 3HP at either age. Animals younger than 10 days were not employed as too many individuals would have to be pooled for a single measurement of serum gonadotropins by RIA.

To test the sensitivity of the *in vivo* model to the orientation of the hydroxyl group on the third carbon of 3HP, 25 day old intact male rats were treated with a single injection of 3β-hydroxy-4-pregnen-20-one (3βHP; 0, 10 20, 40 80 or 160 μg/100 g BW). Figure 8 shows that, at all doses except 20 μg/100 g BW, 3βHP actually increased serum FSH levels ($p<0.01$) and had no effect on LH. The increase in serum FSH was not consistent and did not appear to be dose-related.

Since 3HP can easily be metabolized *in vivo* by endogenous enzymes the effect of two possible metabolites, 5α-pregnane-3,20-dione and 3α-hydroxy-5α-dihydroprogesterone, was tested. A single injection was given to intact 26 day old male rats at a dose of 0, 5 or 40 μg/100 g BW (Figure 9). Neither steroid affected FSH levels, while 40 μg/100 g BW 5α-pregnane-3,20-dione significantly increased serum LH levels ($p<0.05$).

3.1.2 Prepubertal Gonadectomized Rats

Since 3HP appeared to selectively reduce serum FSH levels in the intact prepubertal male rat, it was of interest to determine whether this FSH-suppressing effect could be elicited in the gonadectomized prepubertal rat, a model where gonadotropin secretion is elevated. Male and female prepubertal rats were gonadectomized and, after two days rest, treated with 4 daily injections of 0, 20, 40, 80 or 160 μg 3HP/100

Figure 7: Effect of a single s.c. injection of 3HP (0, 20, 40 or 80 µg/100 g BW) on serum FSH and LH in 10 day old ( and  , respectively) and 20 day old ( and  , respectively) intact male rats. Animals were treated at the age indicated and sacrificed 24 hr later. RIA measurements were made on duplicate samples of 200 µl. Bars represent the mean±SEM of n=4-8 animals and results are expressed as ng RP-1/ml of serum.

10d, 10 day old rats

20d, 20 day old rats

*p<0.05, **p<0.01, ***p<0.001 compared to vehicle-treated control

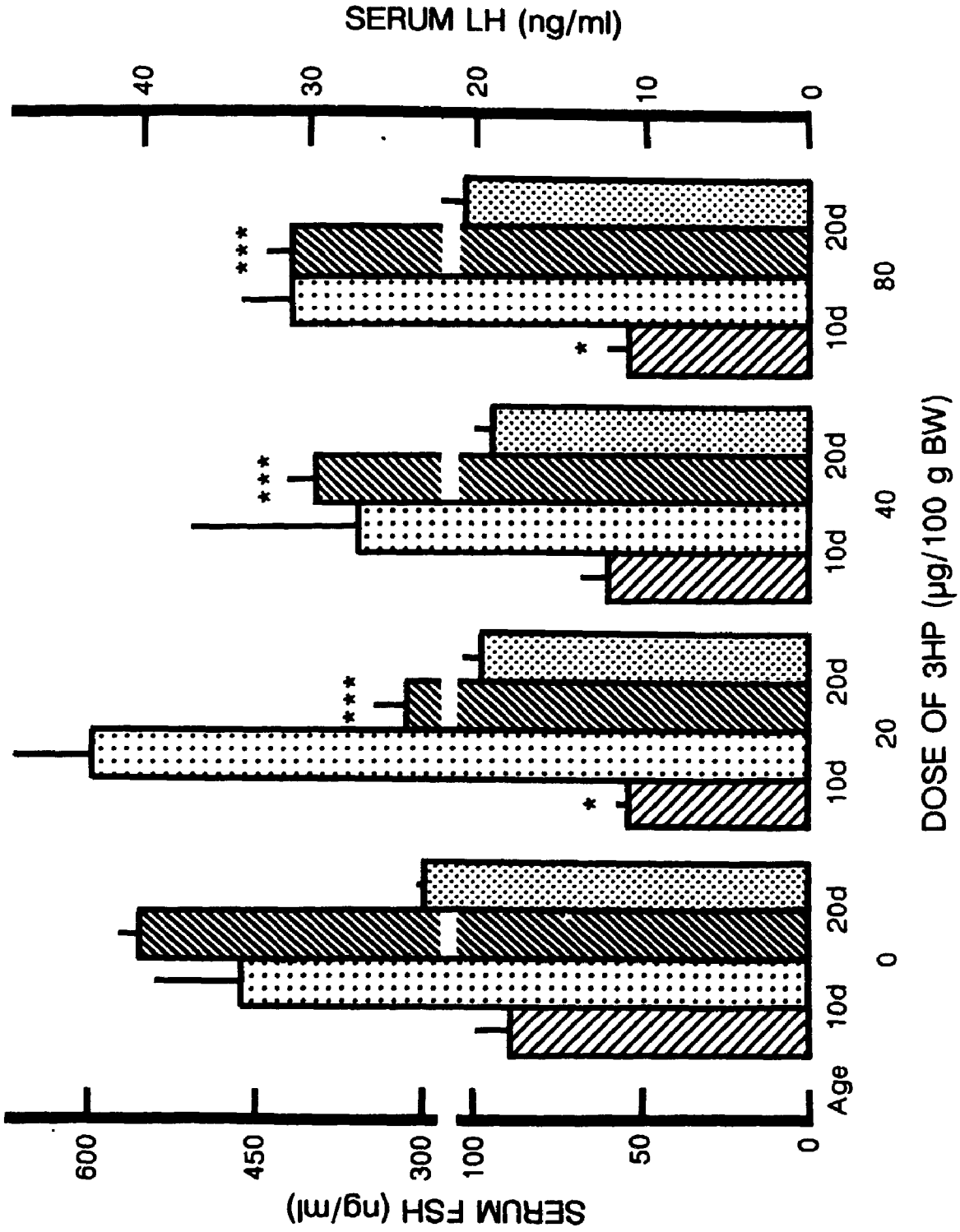

SERUM LH (ng/ml)
40
30
20
10
0
***
*
***
***
*
20d
10d
80
40
20
0
Age
DOSE OF 3HP (µg/100 g BW)
600
450
300
100
50
0
SERUM FSH (ng/ml)

Figure 8: Effect of a single s.c. injection of 3β-hydroxy-4-pregnen-20-one (3βHP) (0, 10, 20, 40, 80 or 160 µg/100 g BW) on serum FSH (▨) and LH (⁘) levels in 25 day old intact male rats. Animals were sacrificed 24 h after injection and RIA determinations made on duplicate 200 µl samples. Bars represent the mean±SEM of n=4-7 animals and results are expresed as ng RP-1/ml of serum.

*p<0.05, **p<0.01 and ***p<0.001 compared to vehicle-treated control

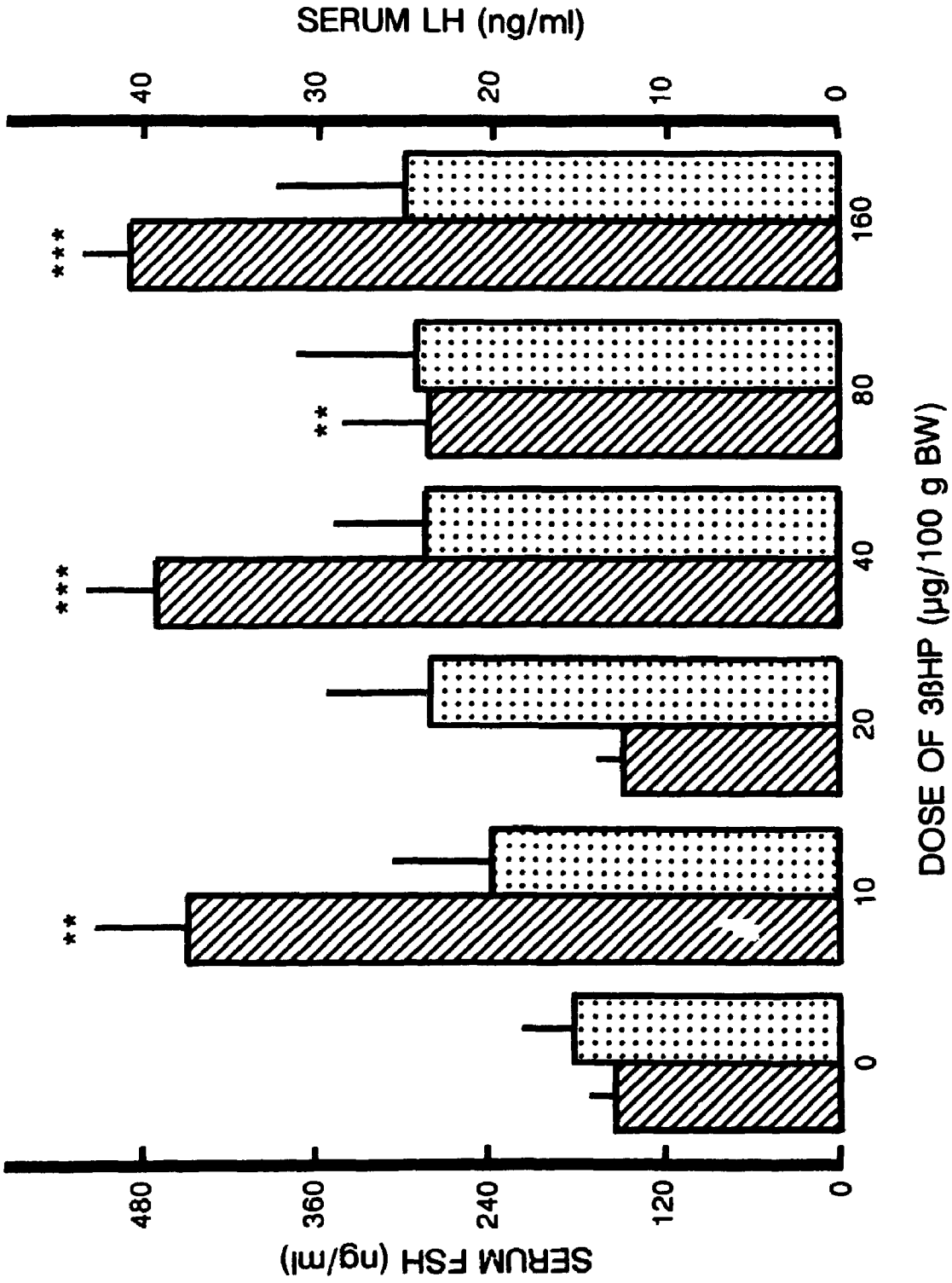
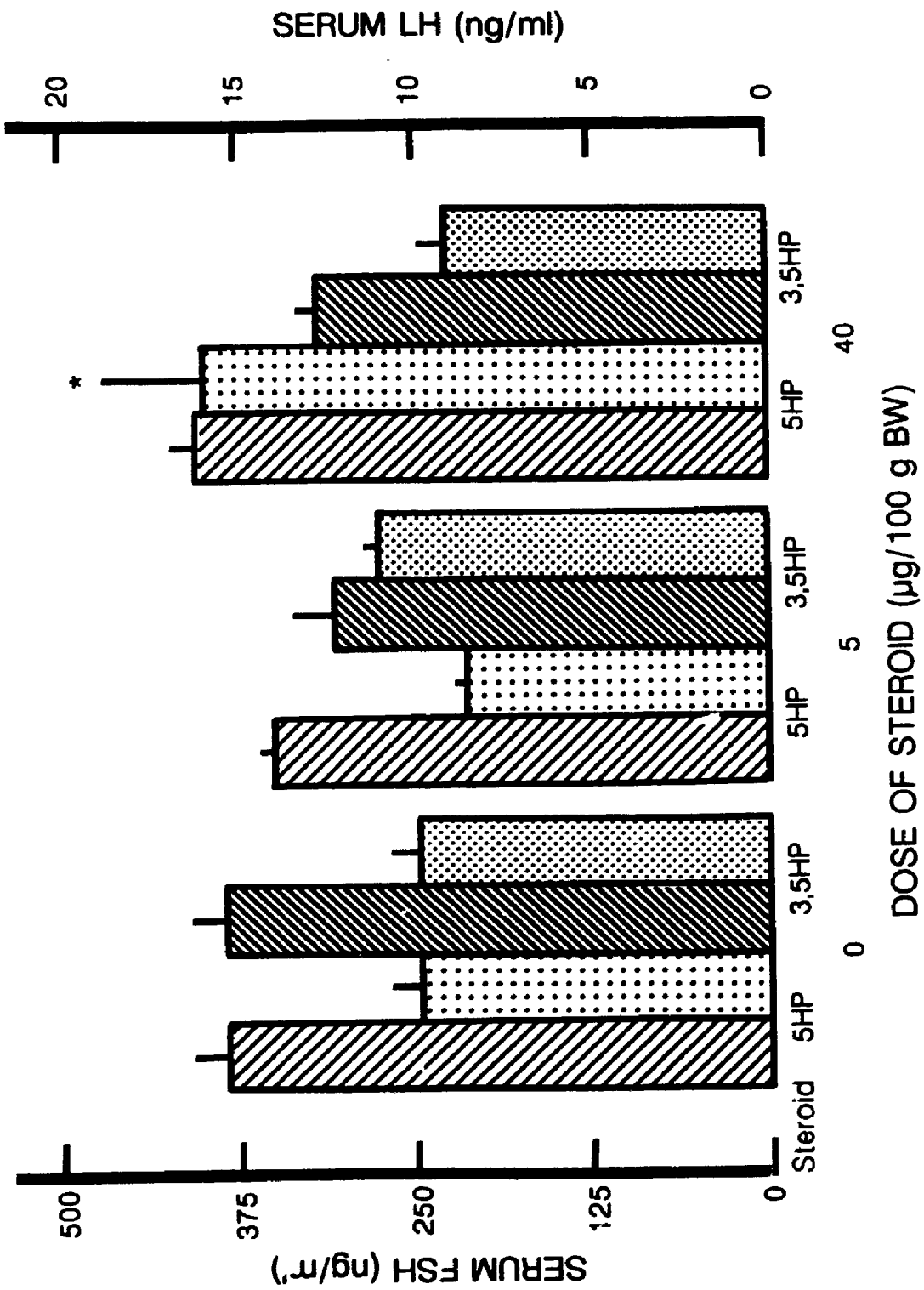

SERUM LH (ng/ml)
40
30
20
10
0
160
80
40
20
10
0
DOSE OF 3βHP (μg/100 g BW)
***
**
***
**
480
360
240
120
0
SERUM FSH (ng/ml)

Figure 9: Effect of 5α-pregnane-3,20-dione ( and ) and 3α-hydroxy-5α-pregnan-20-one ( and ) on serum FSH and LH levels, respectively, in 25 day old intact male rats. A single s.c. injection of either steroid at a dose of 0, 5 or 40 µg/100 g BW was given and serum FSH and LH determined on duplicate 200 µl samples taken 24 hr after treatment. Bars represent the mean±SEM of n=4-7 animals and are expressed as ng RP-1/ml of serum.

5HP, 5α-pregnane-3,20-dione

3,5HP, 3α-hydroxy-5α-pregnane-3,20-dione

*$p<0.05$ compared to vehicle-treated control


SERUM LH (ng/ml)
20
15
10
5
0
*
5HP
3,5HP
5HP
3,5HP
5HP
3,5HP
Steroid
0
5
40
DOSE OF STEROID (µg/100 g BW)
SERUM FSH (ng/ml)
500
375
250
125
0

g BW (females) or 200 μg/100 g BW 3HP (males) (Figure 10). Serum LH levels were not affected by 3HP treatment in males or females although gonadectomy did cause these levels to rise significantly over those of sham-operated controls ($p<0.001$). Serum FSH levels were suppressed by treatment with 20, 80 or 200 μg/100 g BW 3HP in gonadectomized males ($p<0.01$) and by 20, 40 or 80 μg/100 g BW 3HP in gonadectomized females ($p<0.05$). Maximum inhibition of FSH secretion occurred at the lowest dose administered (20 μg/100 g BW) in males and females, to 62% and 56% of gonadectomized control levels, respectively. Gonadectomy significantly increased FSH secretion in both males and females ($p<0.001$).

Due to its labile nature, 3HP was acetylated (as described in Wiebe et al, 1985) in hopes that this would confer stability and a longer biological half-life to the steroid and result in more consistent in vivo results. 3α-Hydroxy-4-pregnen-20-one-3-acetyl (3HPA) was administered daily for 4 days to gonadectomized prepubertal male rats at doses of 0, 0.1, 0.5, 2.5, 12.5 and 62.5 μg/100 g BW. Sham-operated animals received vehicle only. Figure 11 shows that 3HPA selectively inhibited serum FSH levels in a dose-related manner ($p<0.01$; ANOVA) and, at a dose of 62.5 μg/100g BW, reduced FSH levels to near that of the sham-operated control (44% of gondectomized control). Serum LH was not affected by treatment with 3HPA ($p=0.2$; ANOVA).

A comparison of the specific effect of 3HP with the well-known gonadotropin-suppressing effects of a few other gonadal steroids was made. 17β-estradiol, dihydrotestosterone and 17α-hydroxyprogesterone were administered to gonadectomized prepubertal male rats at 0 (control for all groups), 2.5 or 62.5 μg/100 g BW for 4 days and sacrificed on

Figure 10: Effect of 3HP on serum gonadotropin levels in gonadectomized male and female prepubertal rats. Animals were gonadectomized at 23 (male) or 24 (female) days of age and allowed two days rest. Treatment began on the third day following surgery and continued for a total of 4 daily s.c. injections of 0, 20, 40, 80 and 160 µg 3HP/100 g BW (female) or 200 ug 3HP/100 g BW (male). Sham-operated animals were treated with saline vehicle only (0) for the duration of the experiment. Animals were sacrificed 24 hr following the last injection and serum FSH (males,  ; females,) and LH (males,  ; females,) levels determined on duplicate 200 µl samples. Bars represent the mean±SEM of n=4-8 animals and results are expressed as ng RP-1/ml of serum.

M, males

F, females

*p<0.05, **p<0.01 and ***p<0.001 compared to vehicle-treated gonadectomized control

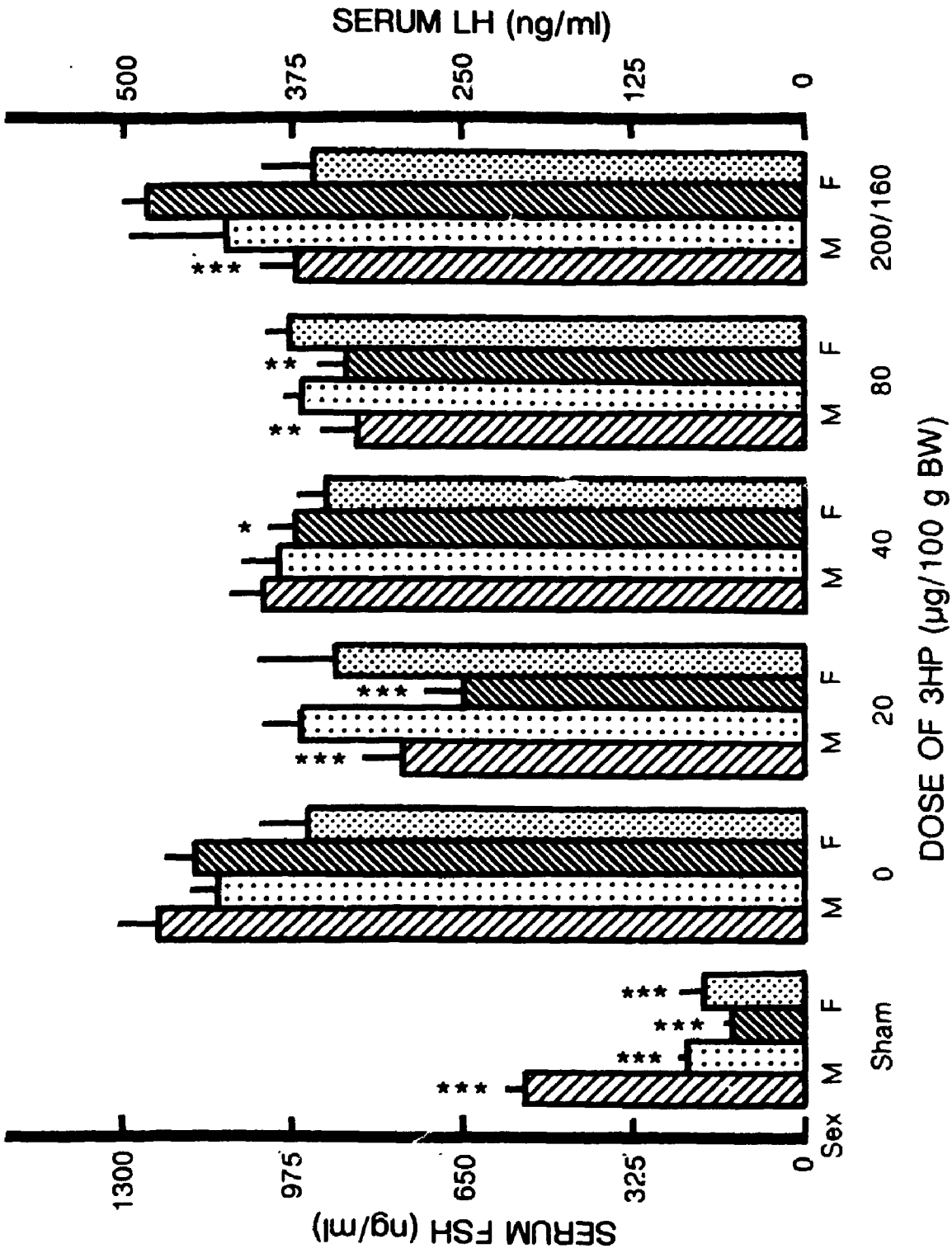
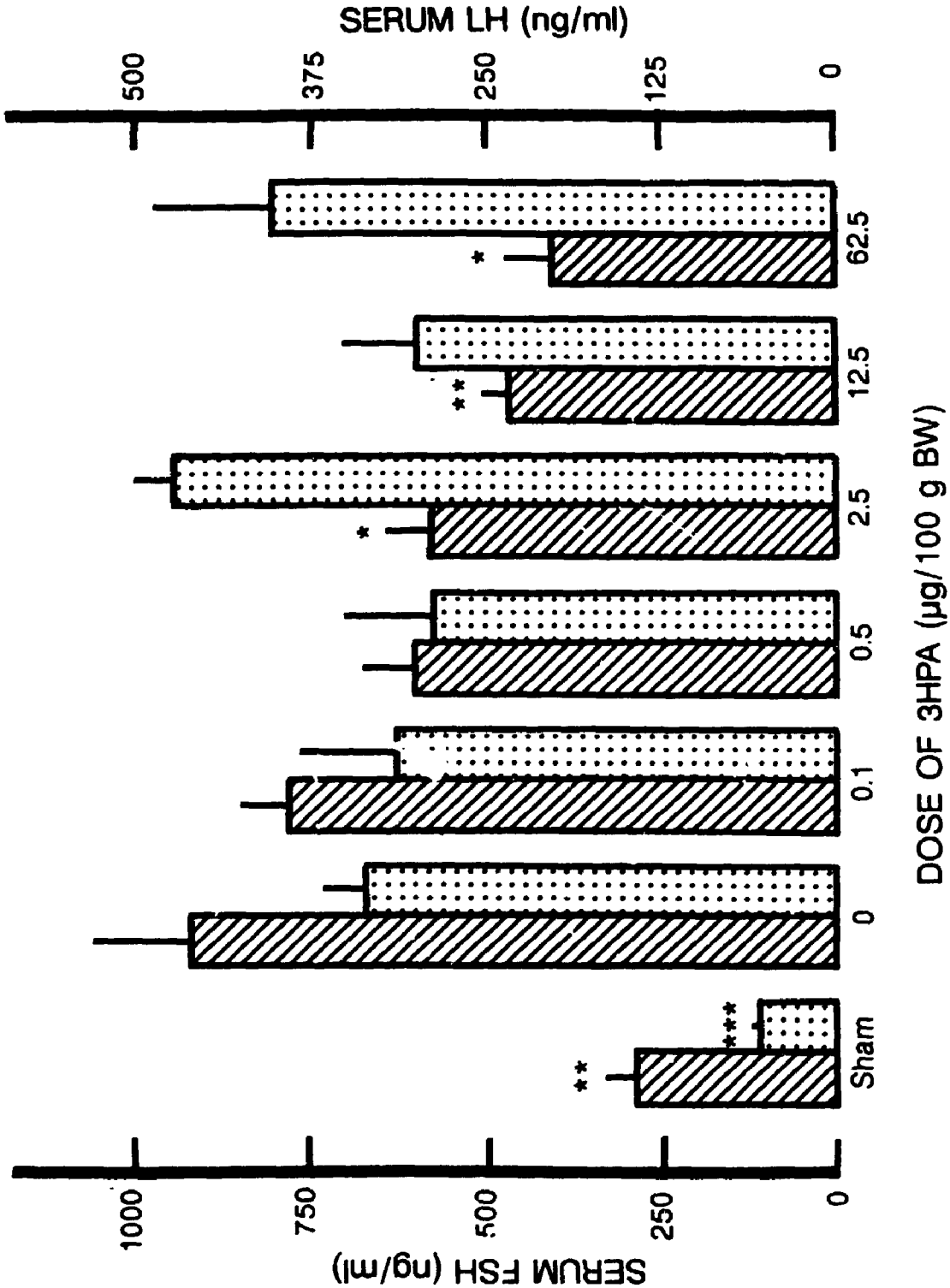

SERUM LH (ng/ml)
500
375
250
125
0
SERUM FSH (ng/ml)
1300
975
650
325
0
Sex
M
F
Sham
0
20
40
80
200/160
DOSE OF 3HP (µg/100 g BW)
***
**
*

Figure 11: Effect of 3α-hydroxy-4-pregnen-20-one-3-acetyl (3HPA) on gonadotropin secretion in gonadectomized, prepubertal male rats. Animals were gonadectomized at 20 days of age and treated with 0, 0.1, 0.5, 2.5, 12.5 or 62.5 µg/100 g BW 3HPA for 4 consecutive days from age 23-26 days. Sham-operated animals were treated with saline vehicle throughout the experiment. Animals were sacrificed 24 hr after the last treatment and serum FSH () and LH () levels were determined on duplicate 200 µl samples. Bars represent the mean±SEM of n=5-7 animals and are expressed as ng RP-1/ml serum. Analysis of variance (ANOVA) showed that 3HP significantly suppressed serum FSH levels ($p<0.01$) and had no effect on serum LH levels ($p=0.2$).

*$p<0.05$ and **$p<0.01$ compared to gonadectomized vehicle-treated control (t-test)


SERUM LH (ng/ml)
500
375
250
125
0
62.5
12.5
2.5
0.5
0.1
0
Sham
DOSE OF 3HPA (µg/100 g BW)
1000
750
500
250
0
SERUM FSH (ng/ml)

the morning of the fifth day. All 3 steroids significantly ($p<0.05$) inhibited serum FSH and LH levels at the highest dose (62.5 µg/100 g BW) (Figure 12). The 17α-hydroxyprogesterone treatment suppressed the level of both gonadotropins at the lower dose (2.5 µg/100 g BW) ($p<0.05$). None of these steroids had a selective effect on FSH, nor were they as effective as 3HP at suppressing FSH levels at the lower dose (see Figure 11). Serum LH levels were always suppressed more than FSH by 17β-estradiol, dihydrotestosterone and 17α-hydroxyprogesterone.

3.1.3 Adult Male Rats

Since 3HP proved its efficacy in selectively inhibiting serum FSH levels in the prepubertal intact or gonadectomized male rat, it was of interest to determine the effect of 3HP on serum gonadotropin levels in the adult. Intact 55 day old male rats received a single injection of 12.5 µg/100 g BW of either 3HPA or 3βHPA and were sacrificed the following morning. Figure 13 shows that, while no significant effects on gonadotropin levels were observed, 3HPA did decrease serum FSH levels by 25% with no observable effect on LH while 3βHPA increased FSH secretion by 35%, again with no effect on LH. At 55 days of age, these animals are just becoming sexually mature.

Another approach in the adult male rat was taken. Male rats were gonadectomized at 90 days of age and allowed 2 days rest. Intact animals of the same age were also used. All animals were treated with 62.5 µg/100 g BW 3HPA (the most effective dose in the prepubertal castrate model-Figure 10) from age 93-96 days and sacrificed at 97 days of age. Figure 14 is a presentation of the results. 3HPA suppressed the rise in serum FSH in the gonadectomized adults to approximately 15% of the

Figure 12: Effect of 17β-estradiol ( , ), dihydrotestosterone ( , ) and 17α-hydroxy-progesterone ( , ) on serum FSH and LH levels, respectively, in gonadectomized prepubertal male rats. Rats were gonadectomized at 22 days of age, treated from days 25-28 with 0 (control for all groups), 2.5 or 62.5 μg/100 g BW of the indicated steroid and sacrificed 24 hr after the last injection. Duplicate RIA measurements were made on 200 μl samples of serum. Bars represent the mean±SEM of n=3-5 animals and are expressed as ng RP-1/ml of serum.

C, control for all groups

E_2, 17β-estradiol

DHT, dihydrotestosterone

OHP, 17α-hydroxyprogesterone

*p<0.05, **p<0.01 and ***p<0.001 compared to gonadectomized vehicle-treated control (C)

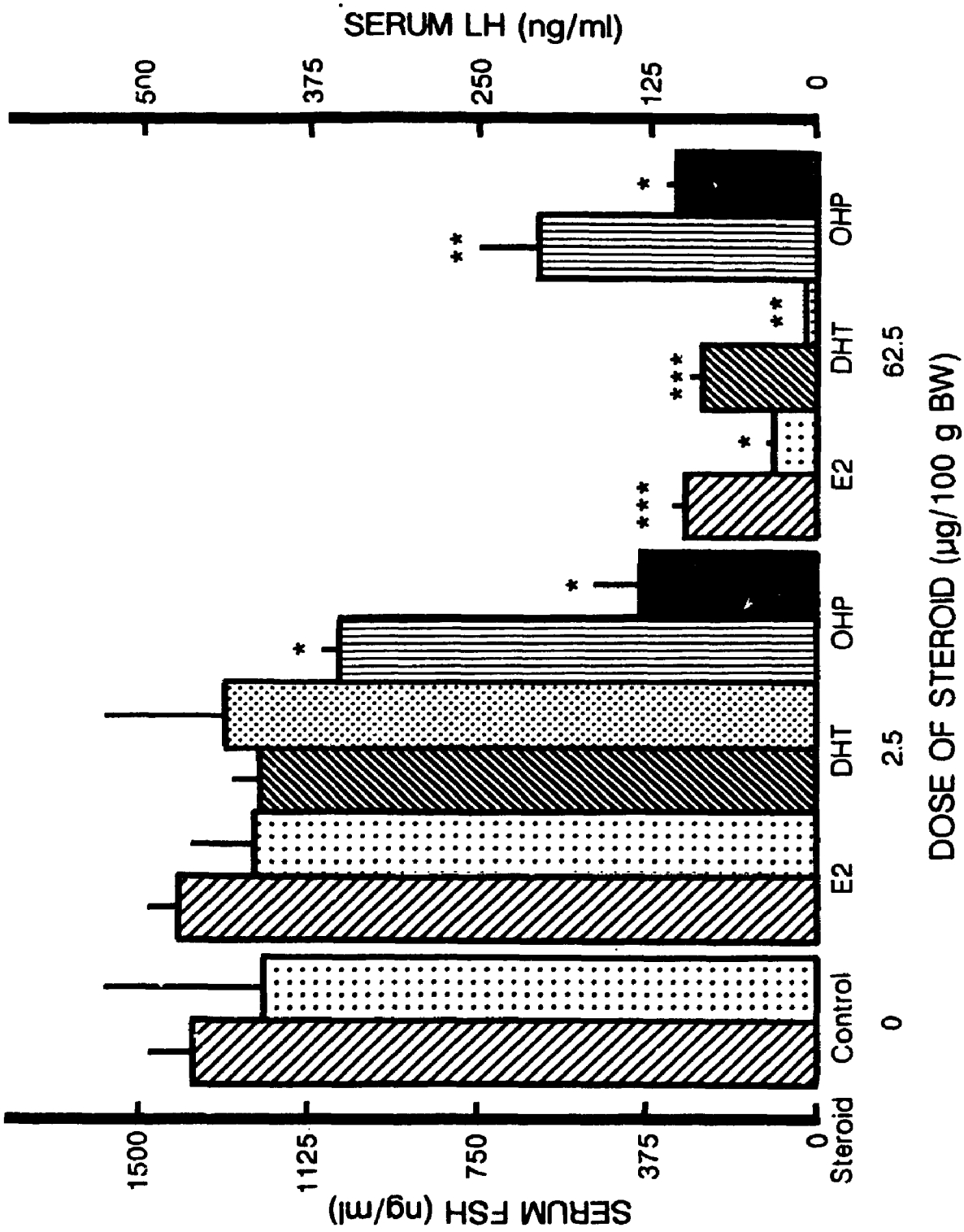

SERUM LH (ng/ml)
500
375
250
125
0
SERUM FSH (ng/ml)
1500
1125
750
375
0
Steroid
Control
E2
DHT
OHP
E2
DHT
OHP
0
2.5
62.5
DOSE OF STEROID (µg/100 g BW)

Figure 13: Effect of 3HPA and 3βHPA on serum FSH () and LH () levels in adult intact male rats. 55 day old male rats were given a single s.c. injection of 12.5 µg/100 g BW of either 3HPA or 3βHPA and sacrificed 24 hr later. Serum gonadotropin levels were measured in duplicate 200 µl samples. Bars represent the mean±SEM of n=4-6 animals and results are expressed as ng RP-1/ml of serum.

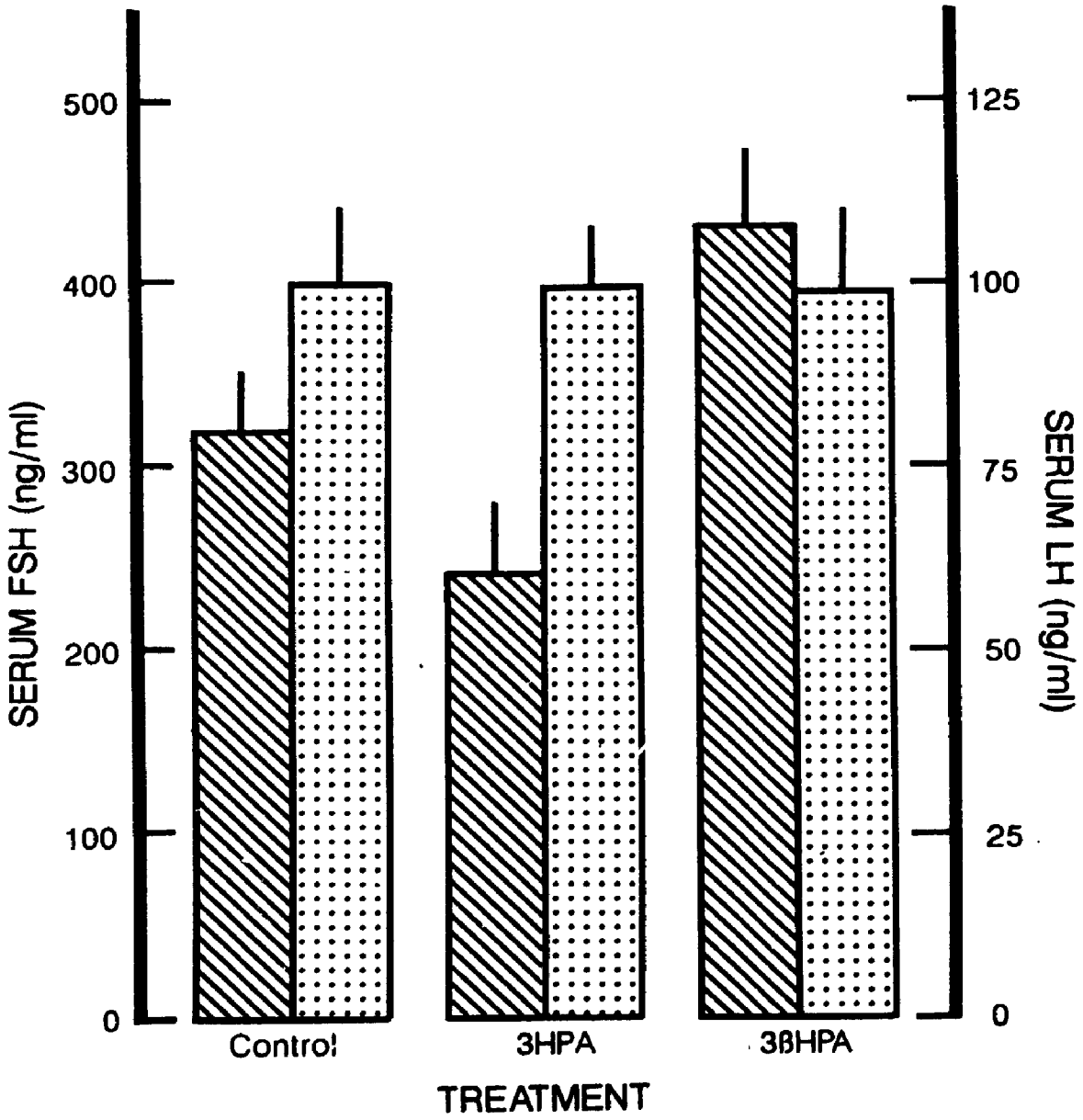

SERUM FSH (ng/ml)
500
400
300
200
100
0
SERUM LH (ng/ml)
125
100
75
50
25
0
Control
3HPA
3βHPA
TREATMENT

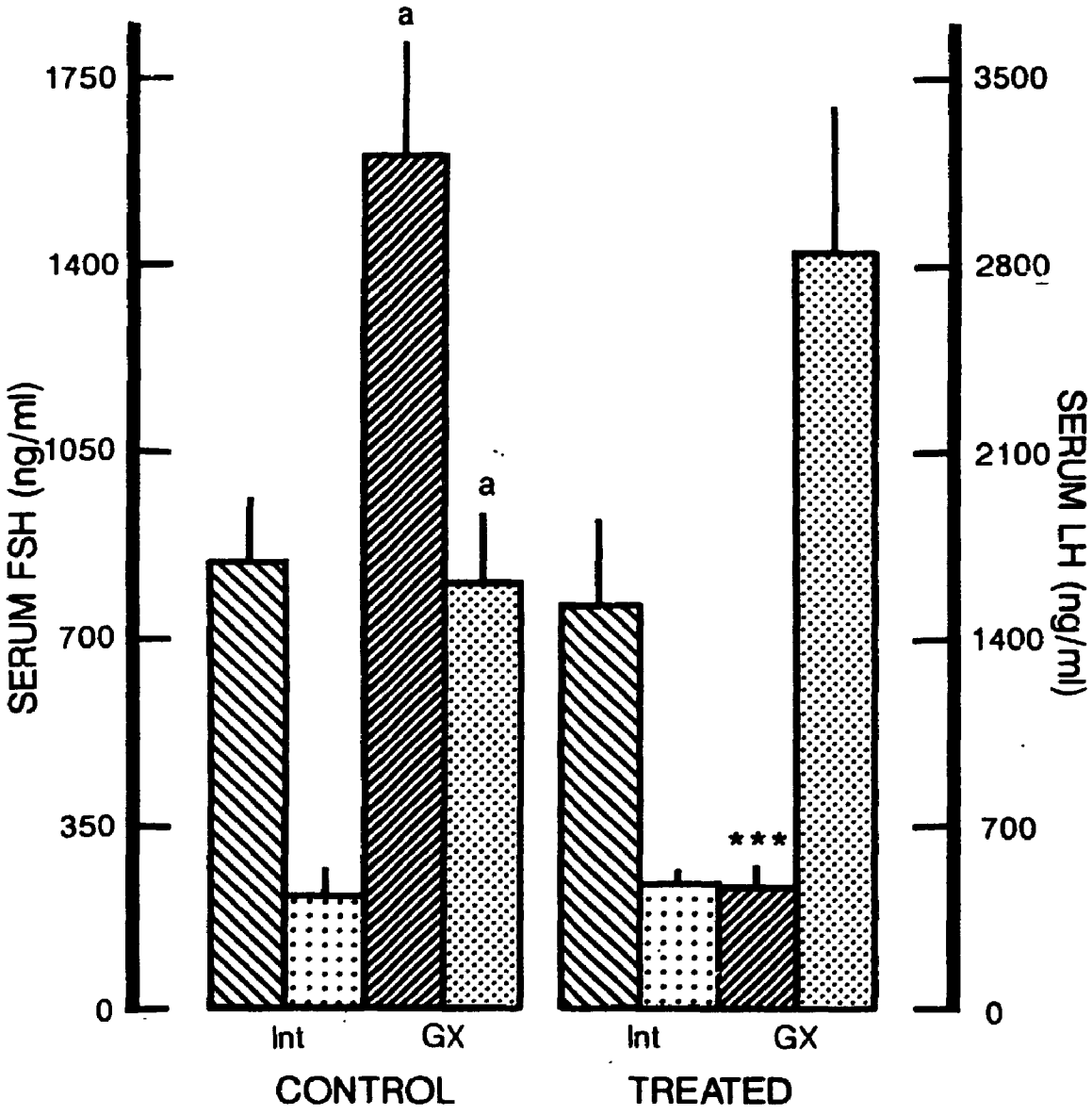
Figure 14: Effect of (3HPA) on serum FSH (▨) and LH (⁘) levels in intact adult male rats and on serum FSH (▨) and LH (⁘) in gonadectomized adult male rats. Animals were gonadectomized at 90 days of age and all animals were treated with daily s.c. injections of 3HPA (62.5 µg/100 g BW) from 93-96 days of age. Rats were sacrificed 24 hr following the last injection and RIA measurement of serum gonadotropins was made on duplicate 200 µl samples. Bars represent the mean±SEM of n=4-6 animals and results are expressed as ng RP-1/ml of serum.

Int, intact

GX, gonadectomized

***$p<0.001$ compared to vehicle-treated control

[a]$p<0.05$ compared to intact vehicle-treated control


a
a
***
1750
1400
1050
700
350
0
3500
2800
2100
1400
700
0
SERUM FSH (ng/ml)
SERUM LH (ng/ml)
Int
GX
Int
GX
CONTROL
TREATED

gonadectomized control level ($p<0.001$) without affecting serum LH. 3HPA did not significantly affect serum gonadotropin levels in the intact adults.

3.2 STUDIES ON PRIMARY CULTURES OF RAT ANTERIOR PITUITARY CELLS

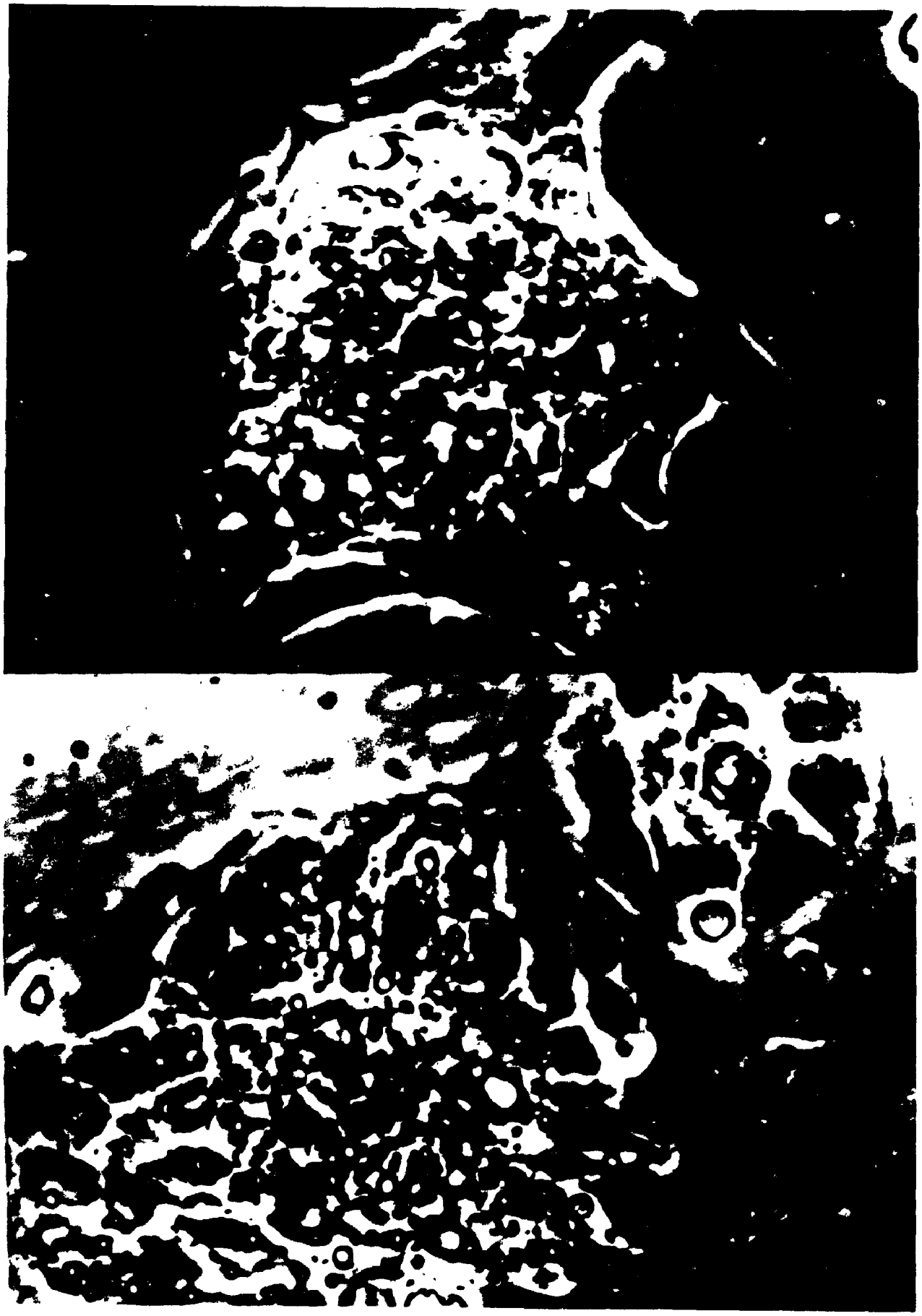
In order to further study 3HP suppression of FSH secretion in the rat, primary cultures of anterior pituitary cells were employed. This system allows for a thorough investigation of 3HP action under controlled conditions and requires fewer animals for each experiment than are required *in vivo*. Typical cultures are shown in Plate 1.

3.2.1 FSH-Suppressing Effect of 3HP In Vitro

Primary cultures of anterior pituitary cells were prepared from mature male and female rats and incubated with various doses of luteinizing hormone-releasing hormone (LHRH) for 4 hr. Cultured anterior pituitary cells from female rats respond better, and at a lower dose, to LHRH than do those from male rats (Figure 15). Figure 15 shows that FSH and LH secretion was maximally stimulated ($p<0.001$) (300-600% of untreated control) by a concentration of 10 nM LHRH in primary cultures of female pituitary cells. Gonadotropin secretion in primary cultures of male pituitary cells was increased by only 70% ($p<0.05$) after treatment with 30 nM LHRH (Figure 15).

In an attempt to confirm *in vivo* results with 3HP, primary cultures of anterior pituitary cells from male rats were prepared and treated with 5 nM 3HP and/or 30 nM LHRH and incubated for 4 or 24 hr after treatment. 3HP selectively inhibited basal FSH secretion to 57% ($p<0.05$) and 75% ($p<0.01$) of control at 4 and 24 hr, respectively (Figure 16).

Plate 1: Typical rat anterior pituitary cells (P) in primary culture. Note that cells with a fibroblast-like morphology ( ) surround clusters of pituitary secretory cells. Photograph taken on a Leitz Diavert Inverted Phase Photomicroscope. Magnification 880X.



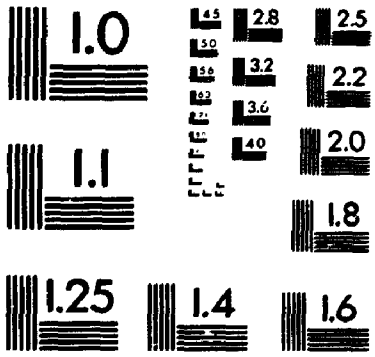

1.0
1.1
1.25
1.4
1.6
1.8
2.0
2.2
2.5
2.8
3.2
3.6
4.0
4.5
5.0
5.6
6.3
7.1

Figure 15: Effect of luteinizing hormone releasing-hormone (LHRH) on FSH (circles) and LH (triangles) secretion in primary cultures of adult male (closed symbols) and female (open symbols) anterior pituitary cells. Cultures were prepared as described (Section 2.4.1), allowed a 72 hr incubation for attachment and then treated with either 0, 0.001, 0.1, 10 and 1000 nM LHRH (female) or 0, 0.003, 0.3, 30 and 3000 nM LHRH (male). Cultures were incubated a further 4 hr at which time medium was removed and gonadotropins measured by RIA. Points represent the mean±SEM of n=4 (female) or 6 (male) replicates and results are expressed as ng RP-2 secreted/mg total culture protein (P)/hr.

*$p<0.05$, **$p<0.01$ and ***$p<0.001$ compared to control (C)

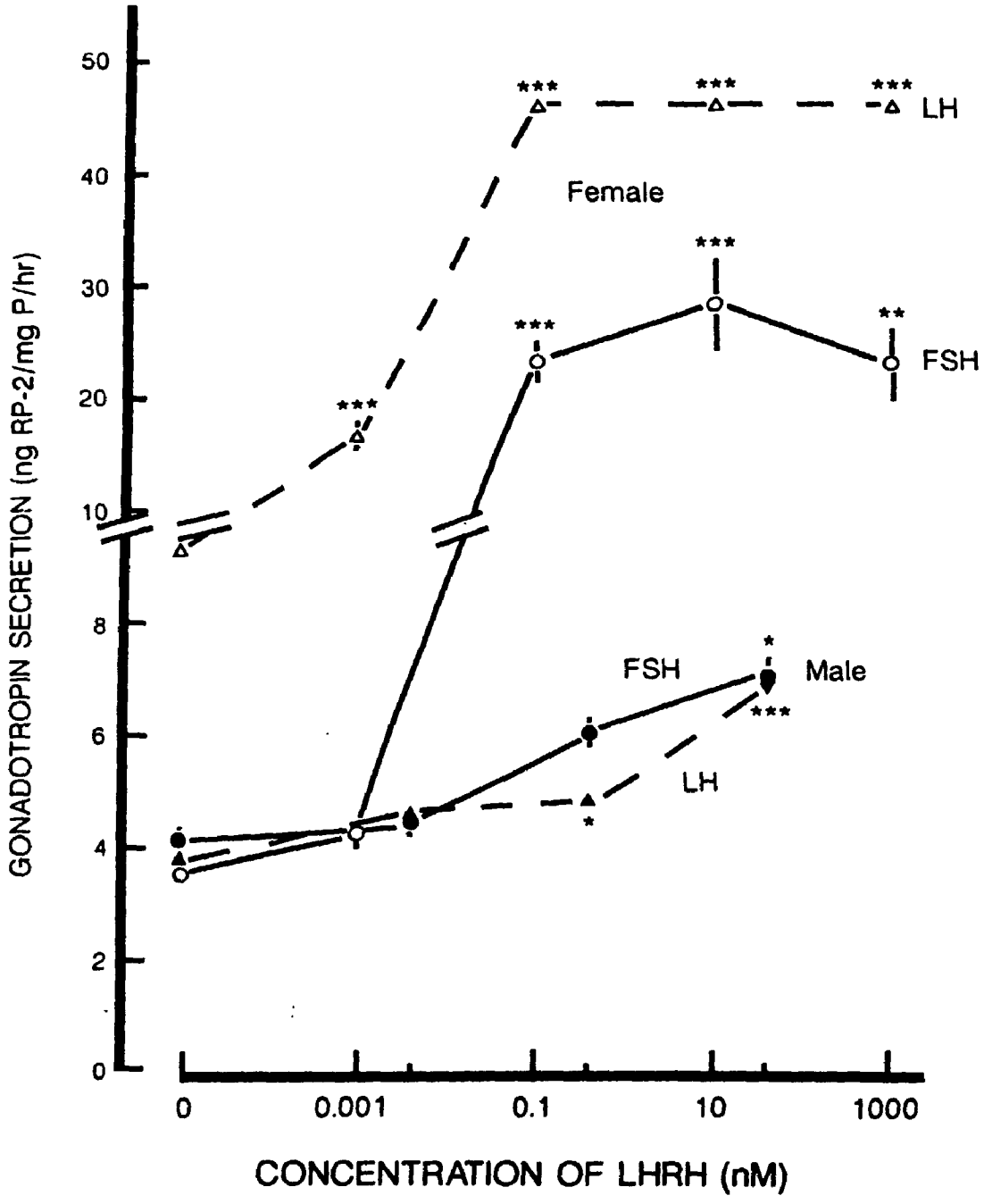

GONADOTROPIN SECRETION (ng RP-2/mg P/hr)
50
40
30
20
10
8
6
4
2
0
LH
Female
FSH
FSH
Male
LH
0
0.001
0.1
10
1000
CONCENTRATION OF LHRH (nM)

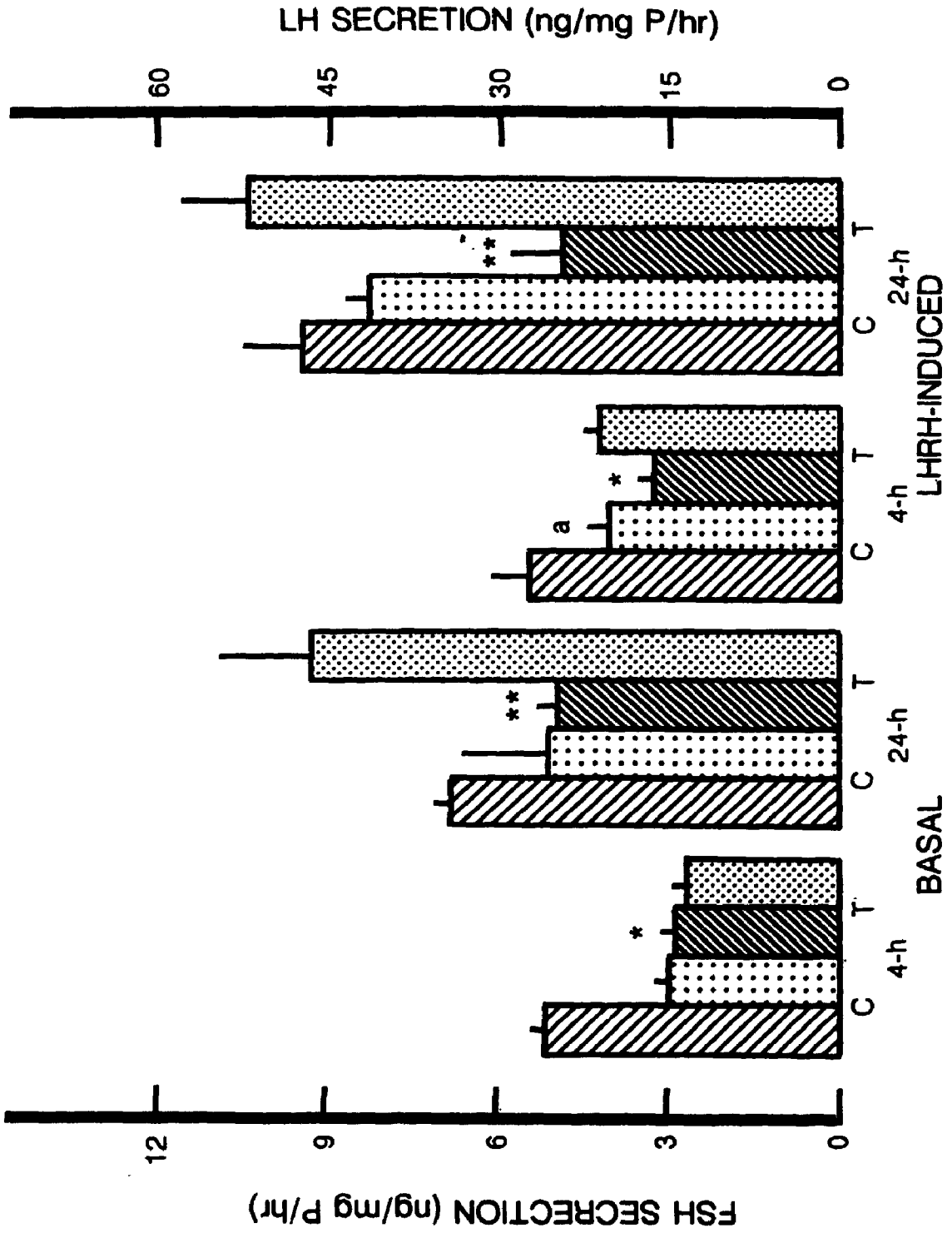
Figure 16: Effect of 3HP (5 nM) and/or LHRH (30 nM) on FSH (stripes) and LH (dots) secretion in primary cultures of adult male rat anterior pituitary cells after a 4 or 24 hr incubation. Cultures were prepared from 55 day old male rats as described (Section 2.4.1) and incubated 72 hr before treatment. Treatments were performed as described (Section 2.4.5) and incubation continued for 4 or 24 hr at which time medium was collected for analysis of gonadotropins by RIA. Bars represent the mean±SEM of n=6 replicates and results are expressed as ng RP-2 secreted/mg total culture protein (P)/hr.

*$p<0.05$ and **$p<0.01$ compared to respective control

[a] $p<0.05$ compared to basal control

C:Control

T:3HP-treated


LH SECRETION (ng/mg P/hr)
60
45
30
15
0
C
T
24-h
LHRH-INDUCED
4-h
a
*
**
BASAL
12
9
6
3
0
FSH SECRETION (ng/mg P/hr)

LHRH-induced FSH release was suppressed by 34% ($p<0.05$) and 48% ($p<0.01$) at 4 and 24 hr, respectively, by 3HP. LH secretion was not affected by 3HP in any of the experimental treatments. Note that 30 nM LHRH failed to elicit a significant increase in FSH secretion at either time period in these cultures, although LH secretion was stimulated by LHRH after a 4 hr incubation ($p<0.05$).

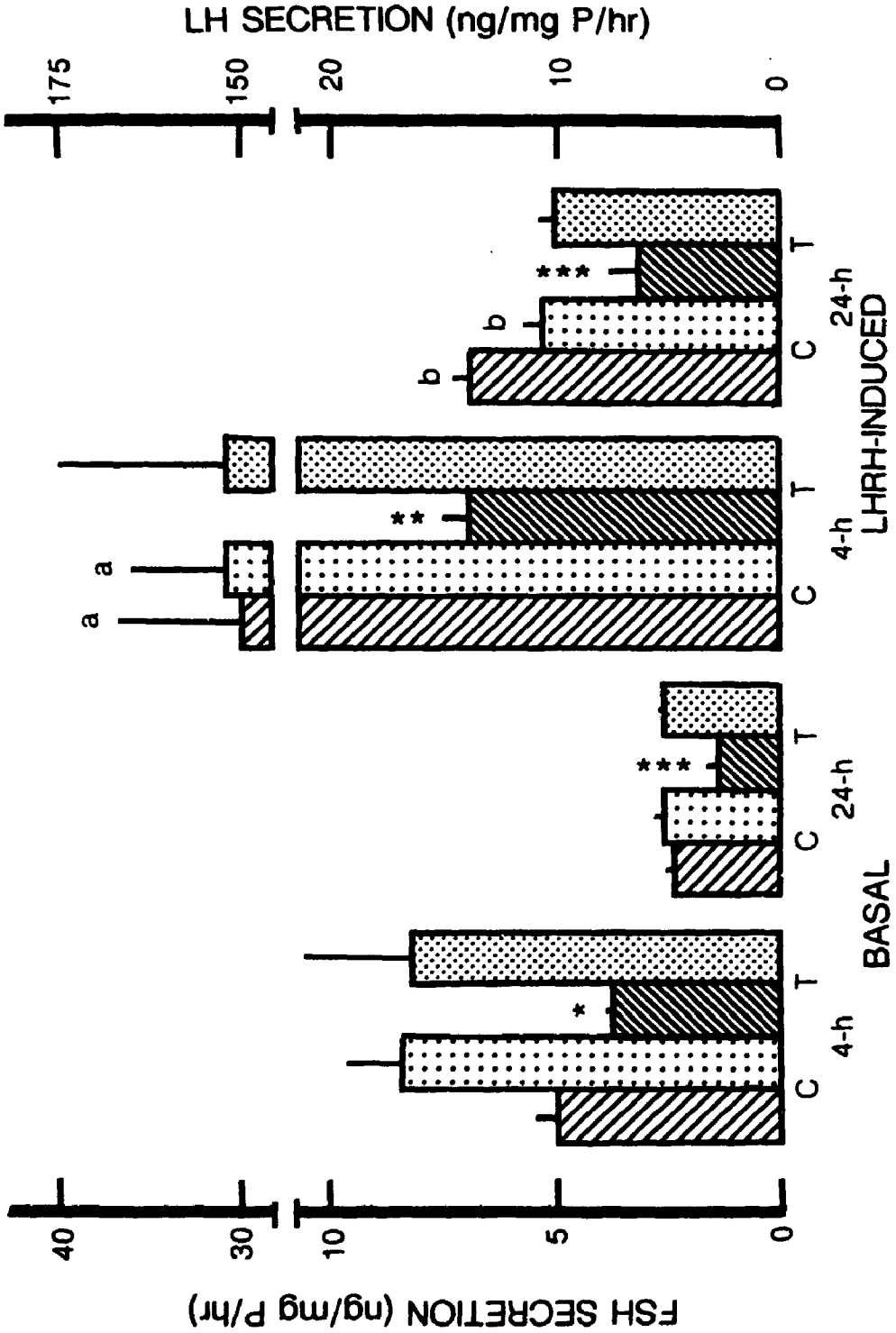
Similar results were obtained with anterior pituitary cells from adult female rats. Figure 17 shows that basal FSH secretion was significantly suppressed to 75% ($p<0.05$) and 63% ($p<0.001$) of the control level, 4 and 24 hr, respectively, after treatment with 10 nM 3HP. Unlike the results from the experiment with cultured male pituitary cells (above), cultures derived from adult females showed a significant surge in secretion of both gonadotropins after a 4 ($p<0.05$) or 24 hr ($p<0.001$) incubation with 10 nM LHRH (Figure 17). Treatment with 10 nM 3HP, in addition to the LHRH, decreased detectable FSH in the medium by 77% ($p<0.01$) and 58% ($p<0.001$) at 4 and 24 hr, respectively, after treatment. A comparison between 3HP-treated and control groups of the secreted LH shows these levels to be nearly identical (Figure 17).

Since cultures of anterior pituitary cells derived from adult female rats responded with a larger and more consistent increase in gonadotropin secretion after challenge by LHRH (Figures 15 and 17) than those from males (Figures 15 and 16), and showed a well-defined, selective reduction of FSH secretion in response to 3HP (Figure 17), female-derived cultures were routinely employed in all subsequent experiments. All incubations were subsequently carried out for 24 hr after treatment since the overall amounts of FSH and LH secreted into the medium were greater at 24 hr than at 4 hr and were therefore more

Figure 17: Effect of 3HP (10 nM) and/or LHRH (10 nM) on FSH (stripes) and LH (dots) secretion in primary cultures of adult female rat anterior pituitary cells after 4 or 24 hr of incubation. Cultures were prepared from 250-300 g females as described (Section 2.4.1) and inubated 72 hr before use. Treatments were carried out as described (Section 2.4.5) and medium sampled after 4 or 24 hr of incubation for analysis of gonadotropins by RIA. Bars represent the mean±SEM of n= 4-6 replicates and results are expressed as ng RP-2 secreted/mg total culture protein (P)/hr.

*$p<0.05$, **$p<0.01$ and ***$p<0.001$ compared to respective control

$^{a}p<0.05$ and $^{b}p<0.001$ compared to basal control


LH SECRETION (ng/mg P/hr)
175
150
20
10
0
LHRH-INDUCED
BASAL
4-h
24-h
C
T
a
a
b
b
*
**
***
***
40
30
10
5
0
FSH SECRETION (ng/mg P/hr)

reliably measured by RIA.

Cultured female anterior pituitary cells were treated with a broad dose range of 3HP (10^{-16}-10^{-4} M) and medium was sampled after a 24 hr incubation. The results are shown in Figure 18. All doses of 3HP inhibited FSH release ($p<0.001$; ANOVA). Maximum suppression of FSH secretion was seen at a dose of 10^{-8} M (10 nM) where FSH levels were only 22% ($p<0.001$) of the untreated control. LH secretion was not affected by 3HP ($p=0.95$; ANOVA). In the same experiment, cells were challenged with 10 nM LHRH during treatment with the above-mentioned doses of 3HP. All but the lowest dose used decreased LHRH-induced FSH secretion into the medium ($p<0.001$, ANOVA; Figure 19), with maximal suppression (91%) occurring at a dose of 10^{-12} M 3HP ($p<0.001$). LHRH (10 nM) considerably increased both FSH ($p<0.05$) and LH ($p<0.001$) secretion into the medium as compared with the basal control (Figure 18). LH secretion was not affected by treatment with 3HP ($p=0.15$; ANOVA).

The foregoing results showed that 3HP can selectively inhibit FSH release in vitro, but did not resolve whether 3HP has any effect on gonadotropin synthesis. Table 4 shows the results of measurements of intracellular FSH and LH from the 4 hr incubations of cultured male and female pituitary cells shown in Figures 16 and 17. There is no difference in cell content of either gonadotropin after 3HP treatment in male-derived cultured pituitary cells. Since Figure 16 shows a marked decrease in FSH release in these same cells and there is no accumulation of FSH within these cells, it appears that there is a decrease in synthesis. The same is true of cultured anterior pituitary cells from female rats. A slight reduction in cell gonadotropins is seen after a 4

Figure 18: Effect of 3HP on basal FSH (▨) and LH (⁘) secretion in primary cultures of adult female rat anterior pituitary cells. Cultures were prepared and treated as described (Section 2.4) and incubated for 24 hr after treatment with 0 (C:Control), 10^{-16}, 10^{-14}, 10^{-12}, 10^{-10}, 10^{-8}, 10^{-6} or 10^{-4} M 3HP after which medium was removed for analysis of gonadotropins by RIA. Bars represent the mean±SEM of n=5-6 replicates and results are expressed as ng RP-2 secreted/mg total culture protein (P)/hr. Basal FSH secretion was suppressed by 3HP in a dose-response manner ($p<0.001$; ANOVA). Regression analysis showed the relationship to have an R^2 value of 0.93.

*$p<0.05$, **$p<0.01$ and ***$p<0.001$ compared to control (t-test)

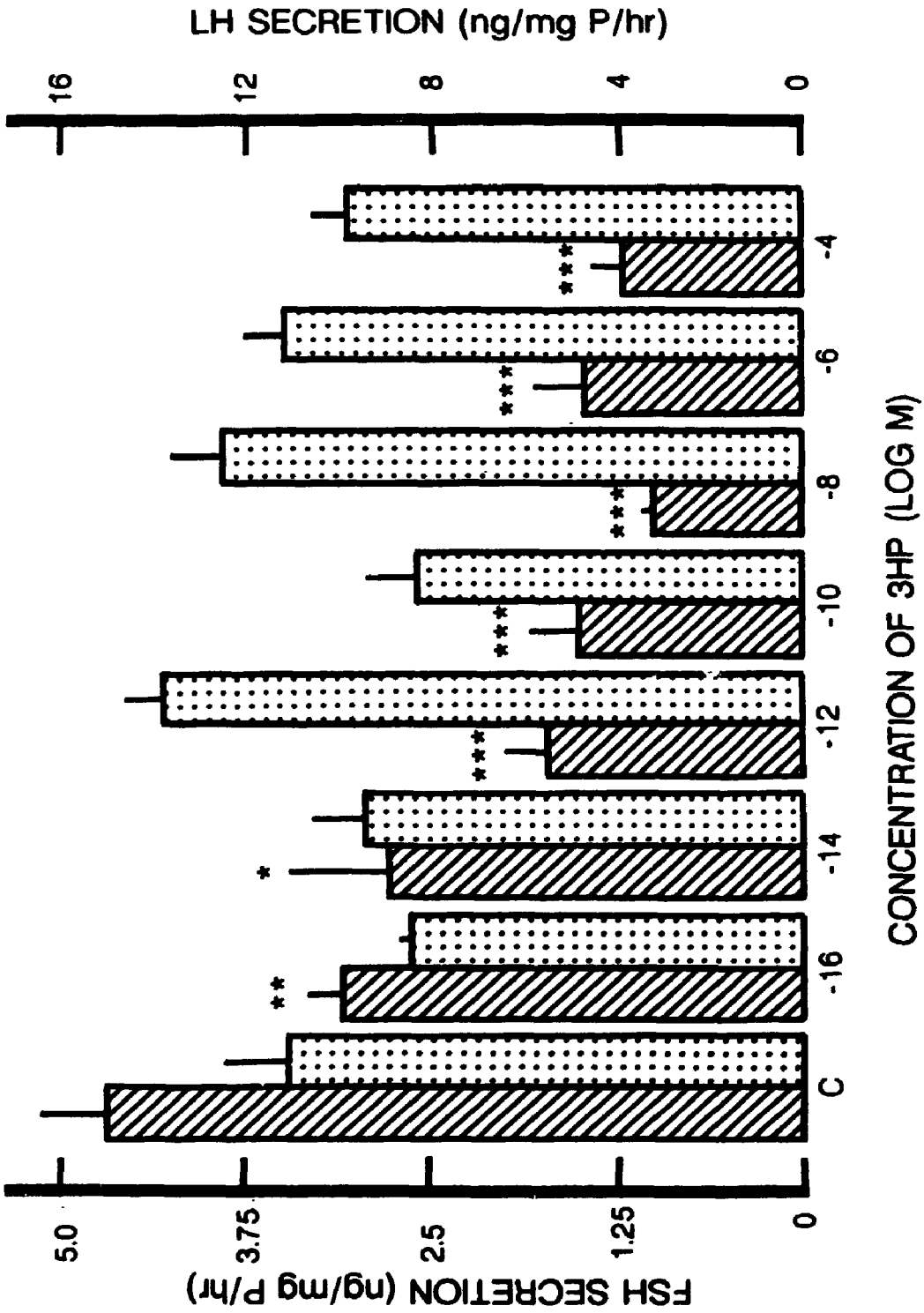

LH SECRETION (ng/mg P/hr)
16
12
8
4
0
-4
-6
-8
-10
-12
-14
-16
C
***
***
***
***
***
*
**
CONCENTRATION OF 3HP (LOG M)
5.0
3.75
2.5
1.25
0
FSH SECRETION (ng/mg P/hr)

Figure 19: Effect of 3HP on LHRH-induced FSH (▨) and LH (⁘) secretion in primary cultures of adult female rat anterior pituitary cells. Cultures were prepared and treated as described (Section 2.4) and incubated for 24 hr after treatment with 0 (C:Control), 10^{-16}, 10^{-14}, 10^{-12}, 10^{-10}, 10^{-8}, 10^{-6} or 10^{-4} M 3HP plus 10 nM LHRH after which medium was removed for analysis of gonadotropins by RIA. Bars represent the mean±SEM of n=4-6 replicates and results are expressed as ng RP-2 secreted/mg total culture protein (P)/hr. LHRH-induced FSH secretion was suppressed by 3HP in a dose-response manner ($p<0.001$; ANOVA). Regression analysis showed the relationship to have an R^2 value of 0.86.

*$p<0.05$ and **$p<0.001$ compared to control (t-test)

[a]$p<0.01$ and [b]$p<0.001$ compared to basal control (Fig. 18; t-test)

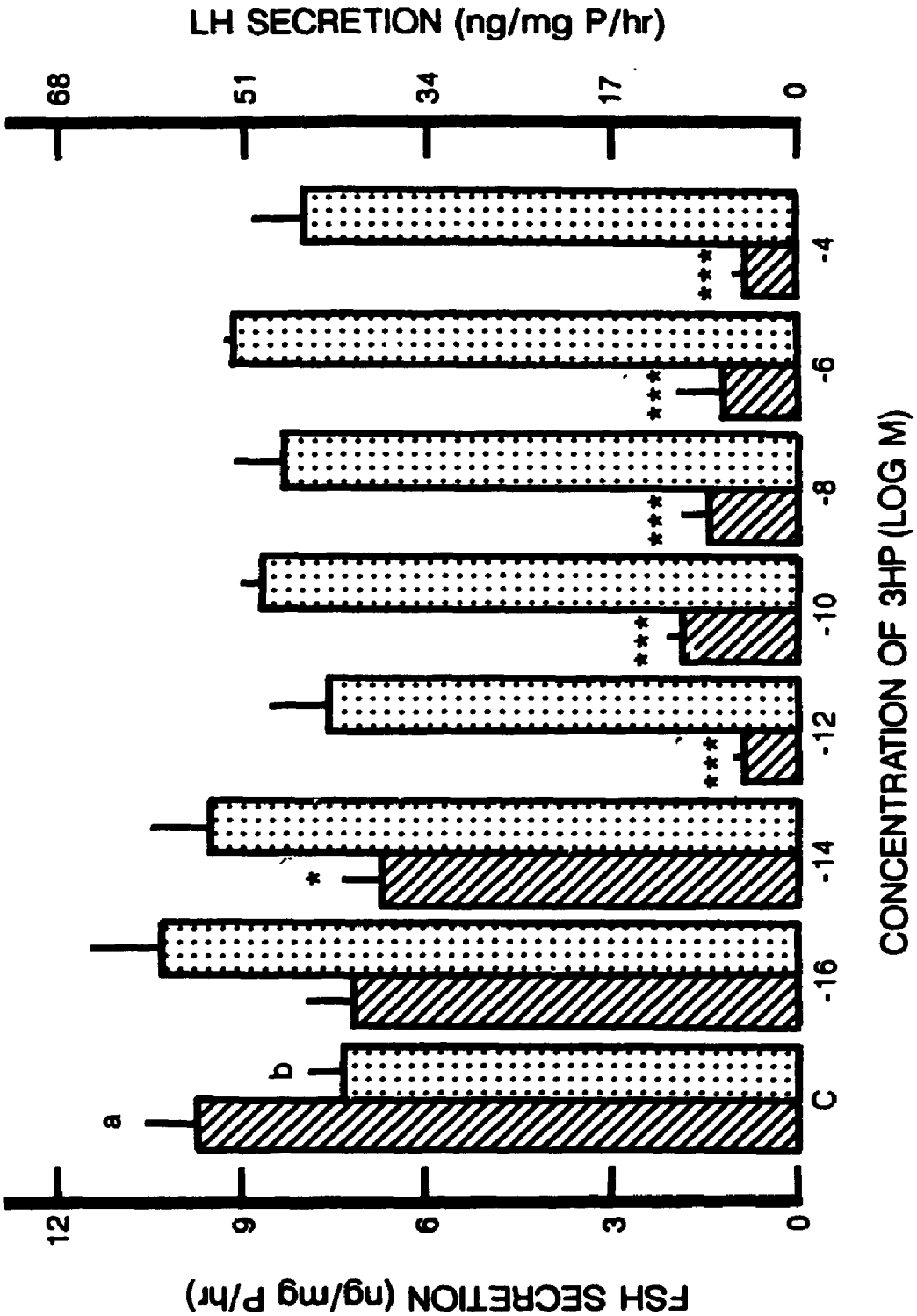

LH SECRETION (ng/mg P/hr)
68
51
34
17
0
-4
-6
-8
-10
-12
-14
-16
C
b
a
12
9
6
3
0
FSH SECRETION (ng/mg P/hr)
CONCENTRATION OF 3HP (LOG M)

Table 4: Effect of 3HP and luteinizing hormone-releasing hormone (LHRH) on cell content of gonadotropins in cultured male and female anterior pituitary cells.

Treatment	Basal		LHRH-Induced	
	FSH	LH	FSH	LH
Male:				
Control	3.51±0.24	20.53± 3.39	4.01±2.00	16.1±2.00
3HP	3.64±0.51	18.50± 4.85	3.30±0.32	16.70±3.09
Female:				
Control	14.99±2.33	155.80±11.27	14.88±0.52	36.50±1.35[a]
3HP	10.70±1.97	119.67±12.47	11.33±1.83	26.36±2.72[b]

Pituitary cells were derived from mature male or female rats and preincubated for 72 hr before treatment. 3HP was used at concentration of 5 nM (male) or 10 nM (female) and LHRH was either 30 nM (male) or 10 nM (female). Samples were taken 4 h after treatment as described (Section 2.4). Results shown are the mean±SEM of n=4-6 replicates and are expressed as ng RP-2/mg total cell protein/hr. These data are from the same experiments shown in Figures 16 and 17.

[a] $p<0.001$ compared to basal control
[b] $p<0.05$ compared to LHRH control

hr treatment with 3HP (Table 4) while a reduction in FSH release occurred (Figure 17), again suggesting that 3HP inhibits FSH synthesis as well as release. Treatment with LHRH significantly reduced intracellular LH levels in female pituitary cells ($p<0.05$) due to increased LH release. Cell levels of FSH in female cultures, and both gonadotropins in male cultures, remained constant indicating a stimulation of gonadotropin synthesis, since more hormone is released under LHRH stimulation, by LHRH in these cells.

3.2.2 Other Gonadal Hormones

3HP is a product of the male gonads, specifically the Sertoli cells. In order to compare 3HP action *in vitro* with other gonadal hormones, anterior pituitary cells fro[illegible] adult female rats were incubated for 24 hr after treatment with various gonadal steroids, with or without challenge by 10 nM LHRH.

Progesterone (also a possible product of 3HP metabolism) was introduced to primary cultures of anterior pituitary cells at concentrations of 0, 0.001, 0.1, 10 and 1000 nM in the medium in the presence or absence of 10 nM LHRH. Figure 20 shows that progesterone significantly increased basal FSH secretion at doses of 0.1 ($p<0.05$) and 1000 nM ($p<0.001$), up to 3.5-fold at the highest dose and, although 10 nM progesterone had no significant effect, the overall trend is toward increasing basal FSH levels. LHRH-induced FSH and basal LH secretion were unaffected by progesterone alone and LHRH-induced LH secretion was significantly ($p<0.01$) reduced by 10 nM progesterone (Figure 20).

The 5α-reduced form of progesterone, 5α-dihydroprogesterone, is a gonadal hormone which may also be a metabolic product of 3HP. Cultures

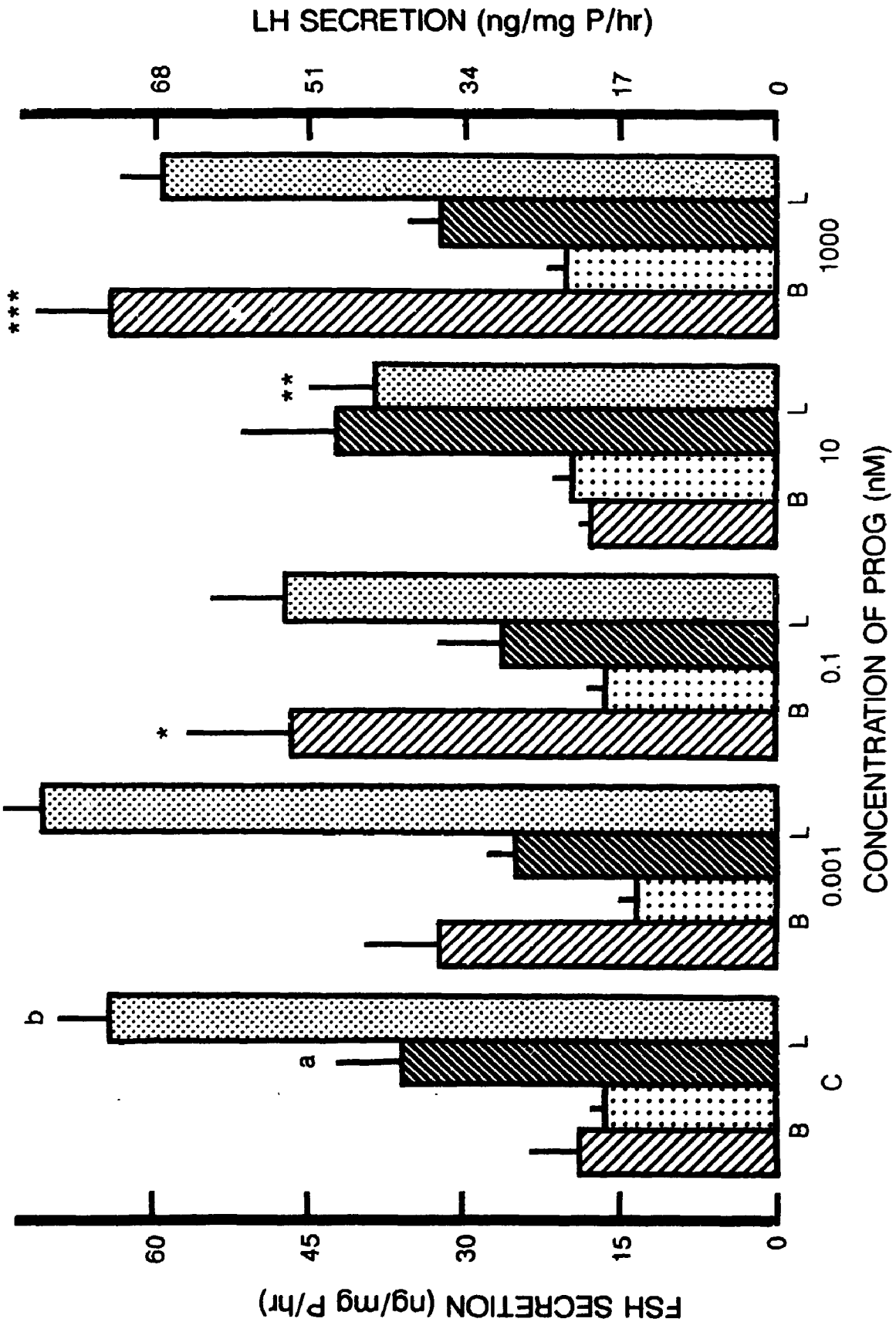
Figure 20: Effect of progesterone (Prog) on basal FSH () and LH () secretion and on LHRH-induced FSH () and LH () secretion by primary cultures of adult female rat anterior pituitary cells. Cultures were prepared and treated as described (Section 2.4) and incubated for 24 hr following treatment with 0 (C:Control), 0.001, 0.1, 10 or 1000 nM Prog and/or 10 nM LHRH. Medium was collected for analysis of gonadotropins by RIA and assayed in duplicate. Bars represent the mean±SEM of n=4-6 replicates and results are expressed as ng RP-2 secreted/mg total culture protein (P)/hr.

b, basal

L, LHRH-induced

*$p<0.05$, **$p<0.01$ and ***$p<0.001$ compared to respective control

$^{a}p<0.05$ and $^{b}p<0.001$ compared to basal control


LH SECRETION (ng/mg P/hr)
68
51
34
17
0
FSH SECRETION (ng/mg P/hr)
60
45
30
15
0
CONCENTRATION OF PROG (nM)
C
0.001
0.1
10
1000
B
L
a
b
*
**
***

of rat anterior pituitary cells treated with 0.001, 0.1, 10 or 1000 nM 5α-dihydroprogesterone showed elevated basal LH levels ($p<0.05$) at all doses while basal FSH secretion was unaffected (Figure 21). 5α-dihydroprogesterone (10 and 100 nM) inhibited LHRH-induced release of FSH ($p<0.01$) and none of the doses influenced LHRH induction of LH secretion.

Testosterone, a product of Leydig cells in the male rat, was introduced into cultures of anterior pituitary cells with or without 10 nM LHRH. Concentrations of 0.1 ($p<0.001$), 10 ($p<0.01$) and 1000 ($p<0.05$) nM testosterone stimulated basal FSH release with the lowest dose having the greatest effect (a 170% increase over basal control) (Figure 22). Testosterone, 10 and 1000 nM, also increased LHRH-induced FSH secretion by about 50% ($p<0.05$). LH secretion showed no response to treatment with testosterone.

Dihydrotestosterone is a metabolite of testosterone and a potent inhibitor of *in vivo* gonadotropin secretion. Anterior pituitary cells were treated with 0, 0.001, 0.1, 10 or 1000 nM dihydrotestosterone and half of these challenged by 10 nM LHRH. None of the doses of dihydrotestosterone tested affected *in vitro* LH secretion (Figure 23). Basal FSH secretion was elevated 30-50% by 10 and 1000 nM dihydrotestosterone ($p<0.05$ and $p<0.01$, respectively) while treatment with 0.1 nM dihydrotestosterone slightly depressed the LHRH-induced release of FSH ($p<0.05$).

Sertoli cells also produce 20α-hydroxy-4-pregnen-3-one. Treatment of cultured pituitary cells with 0, 0.001, 0.1, 10 or 1000 nM 20α-hydroxy-4-pregnen-3-one resulted in a slight suppression of basal LH secretion by 0.1 and 10 nM 20α-hydroxy-4-pregnen-3-one ($p<0.01$) and no

Figure 21: Effect of 5α-dihydroprogesterone (5α-DHP) on basal FSH () and LH () secretion and on LHRH-induced FSH () and LH () secretion in primary cultures of adult female rat anterior pituitary cells. Cultures were prepared and treated as described (Section 2.4) and incubated for 24 hr following treatment with 0 (C:Control), 0.001, 0.1, 10 or 1000 nM 5α-DHP and/or 10 nM LHRH. Medium was collected for analysis of gonadotropins by RIA and assayed in duplicate. Bars represent the mean±SEM of n=4-6 replicates and results are expressed as ng RP-2 secreted/mg total culture protein (P)/hr.

B, basal

L, LHRH-induced

*$p<0.05$ and **$p<0.01$ compared to respective control

$^{a}p<0.05$ and $^{b}p<0.001$ compared to basal control

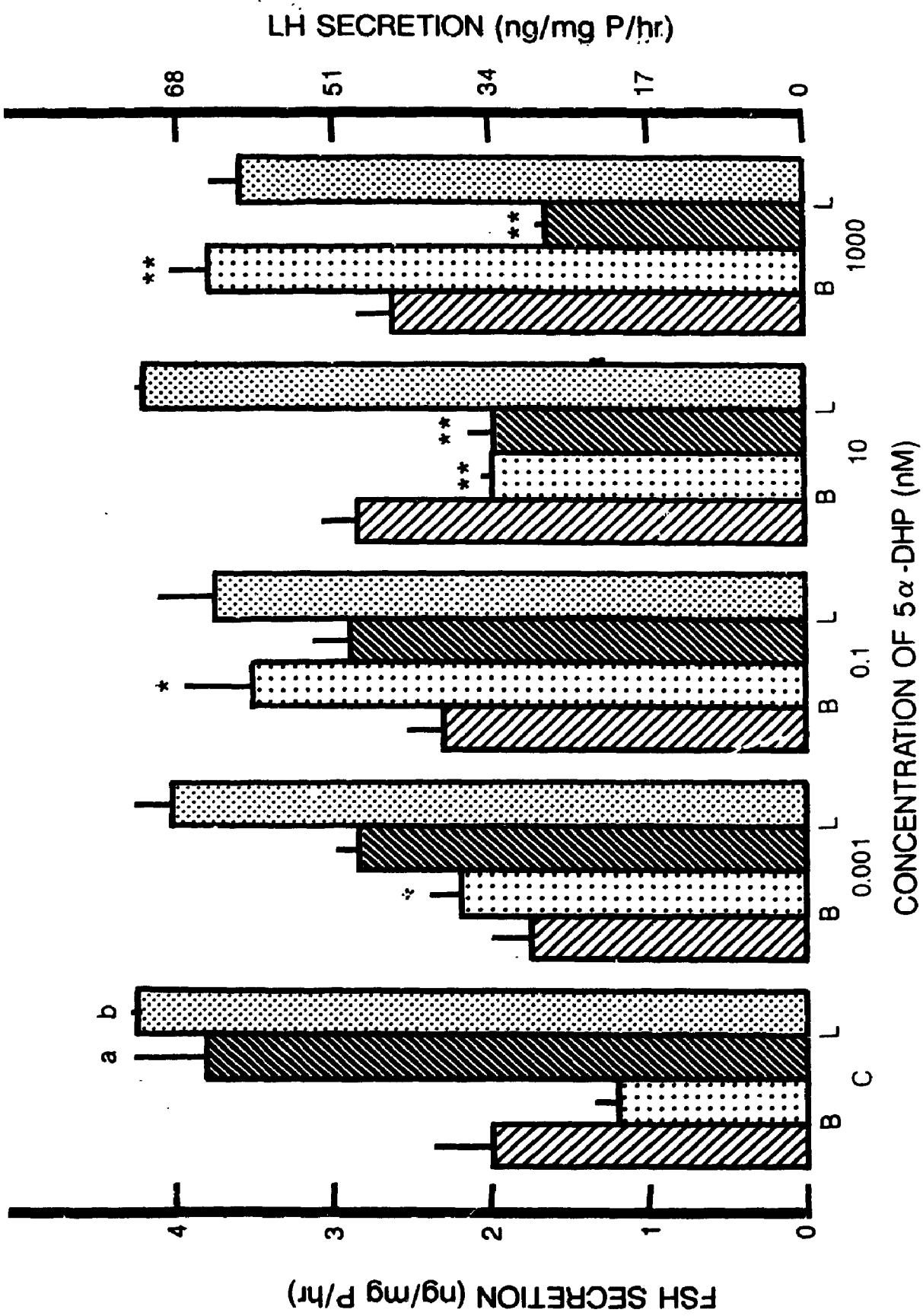

LH SECRETION (ng/mg P/hr)
68
51
34
17
0
FSH SECRETION (ng/mg P/hr)
4
3
2
1
0
a b
B
C
L
0.001
0.1
10
1000
CONCENTRATION OF 5α-DHP (nM)

Figure 22: Effect of testosterone (T) on basal FSH () and LH () secretion and on LHRH-induced FSH ( ) and LH ( ) secretion in primary cultures of adult female rat anterior pituitary cells. Cultures were prepared and treated as described (Section 2.4) and incubated for 24 hr following treatment with 0 (C:Control), 0.001, 0.1, 10 or 1000 nM T and/or 10 nM LHRH. Medium was collected for analysis of gonadotropins by RIA and assayed in duplicate. Bars represent the mean±SEM of n=5-6 replicates and results are expressed as ng RP-2 secreted/mg total culture protein (P)/hr.

B, basal

L, LHRH-induced

*$p<0.05$, **$p<0.01$ and ***$p<0.001$ compared to respective control

[a]$p<0.05$ and [b]$p<0.001$ compared to basal control

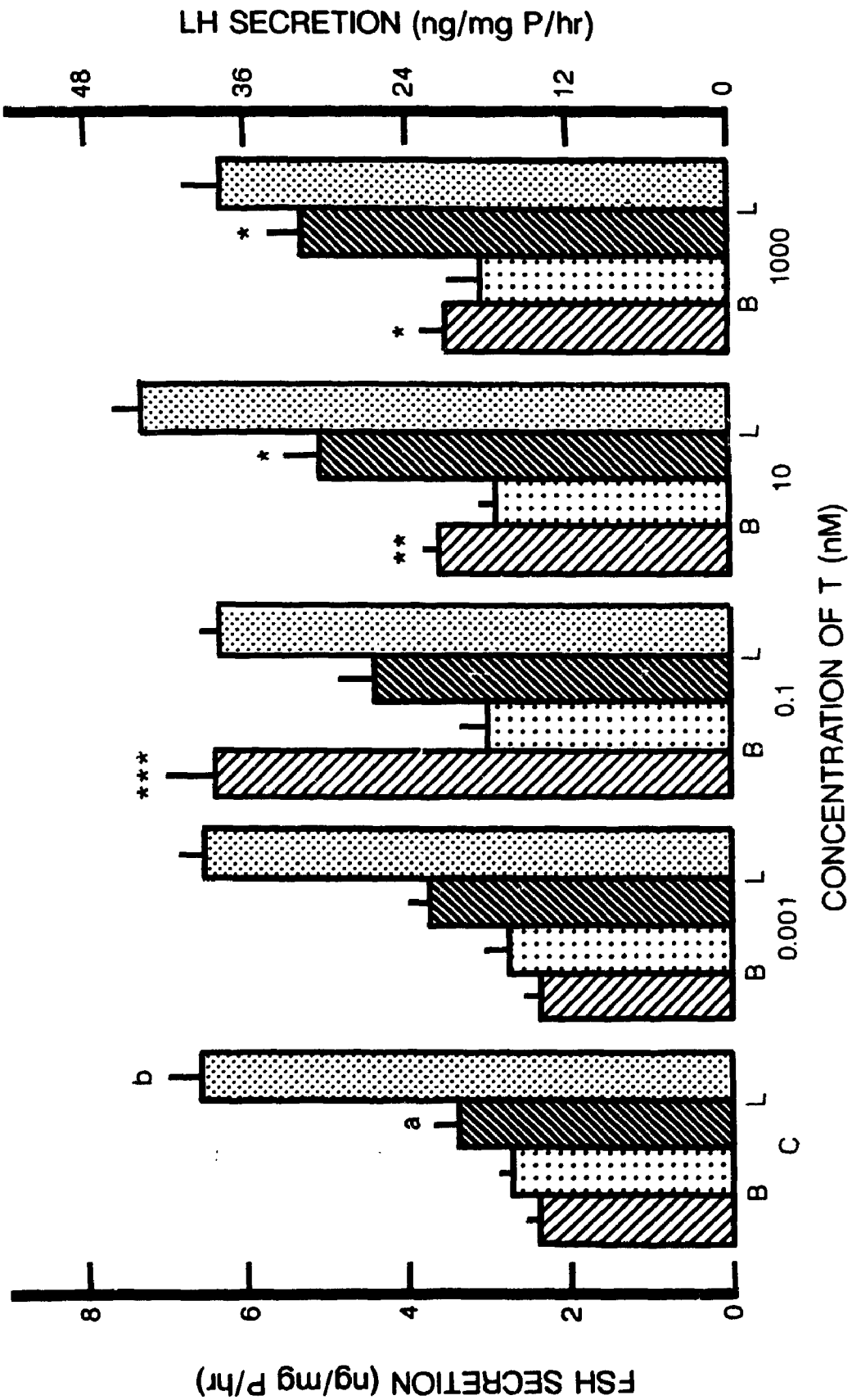

LH SECRETION (ng/mg P/hr)
48
36
24
12
0
L
B
1000
10
0.1
0.001
C
CONCENTRATION OF T (nM)
*
**
***
a
b
8
6
4
2
0
FSH SECRETION (ng/mg P/hr)

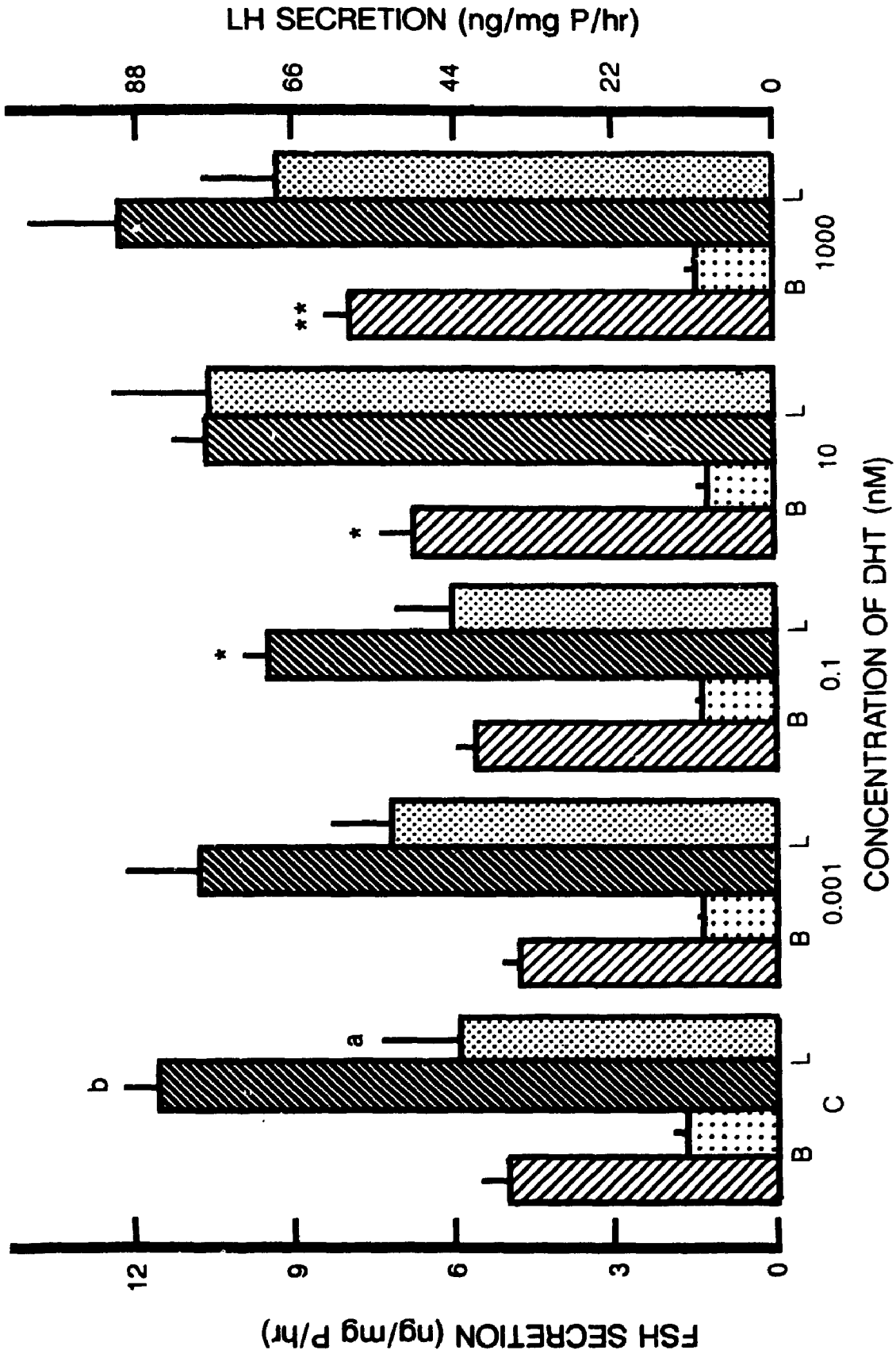
Figure 23: Effect of dihydrotestosterone (DHT) on basal FSH () and LH () secretion and on LHRH-induced FSH () and LH ( ) secretion in primary cultures of adult female rat anterior pituitary cells. Cultures were prepared and treated as described (Section 2.4) and incubated for 24 hr following treatment with 0 (C:Control), 0.001, 0.1, 10 or 1000 nM DHT and/or 10 nM LHRH. Medium was collected for analysis of gonadotropins by RIA and assayed in duplicate. Bars represent the mean±SEM of n=5-6 replicates and results are expressed as ng RP-2 secreted/mg total culture protein (P)/hr.

B, basal

L, LHRH-induced

*$p<0.05$ and **$p<0.01$ compared to respective control

$^{a}p<0.05$ and $^{b}p<0.001$ compared to basal control


LH SECRETION (ng/mg P/hr)
88
66
44
22
0
L
B
1000
10
0.1
0.001
C
**
*
*
a
b
12
9
6
3
0
FSH SECRETION (ng/mg P/hr)
CONCENTRATION OF DHT (nM)

effect on LHRH-induced LH release (Figure 24). Levels of FSH in the medium after stimulation of cells by 10 nM LHRH were further increased by 10 and 1000 nM 20α-hydroxy-4-pregnen-3-one ($p<0.05$) and Figure 24 indicates that this increase appears to be dose-related. None of the 20α-hydroxy-4-pregnen-3-one treatments affected basal FSH.

Estrogen (17β-estradiol) was employed at concentrations of 0, 0.001, 0.1, 10 and 1000 nM in the medium with or without 10 nM LHRH. Table 5 shows the results of this experiment. Basal FSH release was unmodified by 17β-estradiol and only one dose, 10 nM, decreased LHRH-induced FSH release, to 73% of the control level ($p<0.05$). A concentration of 1000 nM 17β-estradiol slightly increased the basal secretion of LH ($p<0.05$) while LHRH-induced LH release was not influenced by 17β-estradiol in this experiment.

Inhibin is a gonadal protein which has been shown to inhibit FSH secretion. Porcine follicular fluid inhibin (PFF-I) was generously supplied by M.R. Sairam and the culture model adjusted to correspond with the technique of Sairam et al (1984). Cultures were prepared from adult female anterior pituitaries as in Section 2.4.1 but the experimental medium used was serum-free M199 supplemented with 4 mM glutamine, 10 nM 17β-estradiol and 1000 nM progesterone. The steroids were added to increase basal secretion of gonadotropins (Sairam et al, 1984). An earlier experiment, using the routine treatment technique described in Section 2.4.5 and used for all previous experiments, showed that the published effects of PFF-I could not be observed (results not shown) without the above-mentioned modifications. The effect of 300 μM PFF-I and/or 10 nM 3HP on basal and LHRH-induced (10 nM) gonadotropin secretion in this model system were compared and the results presented

Figure 24: Effect of 20α-hydroxy-4-pregnen-20-one (20αHP) on basal FSH (▨) and LH (⁘) secretion and on LHRH-induced FSH (▨) and LH (⁘) secretion in primary cultures of adult female rat anterior pituitary cells. Cultures were prepared and treated as described (Section 2.4) and incubated for 24 hr following treatment with 0 (C: Control), 0.001, 0.1, 10 or 1000 nM 20αHP and/or 10 nM LHRH. Medium was collected for analysis of gonadotropins by RIA and assayed in duplicate. Bars represent the mean±SEM of n=5-6 replicates and results are expressed as ng RP-2 secreted/mg total culture protein (P)/hr.

B, basal

L, LHRH-induced

*$p<0.05$ and **$p<0.01$ compared to respective control

$^{a}p<0.05$ and $^{b}p<0.001$ compared to basal control

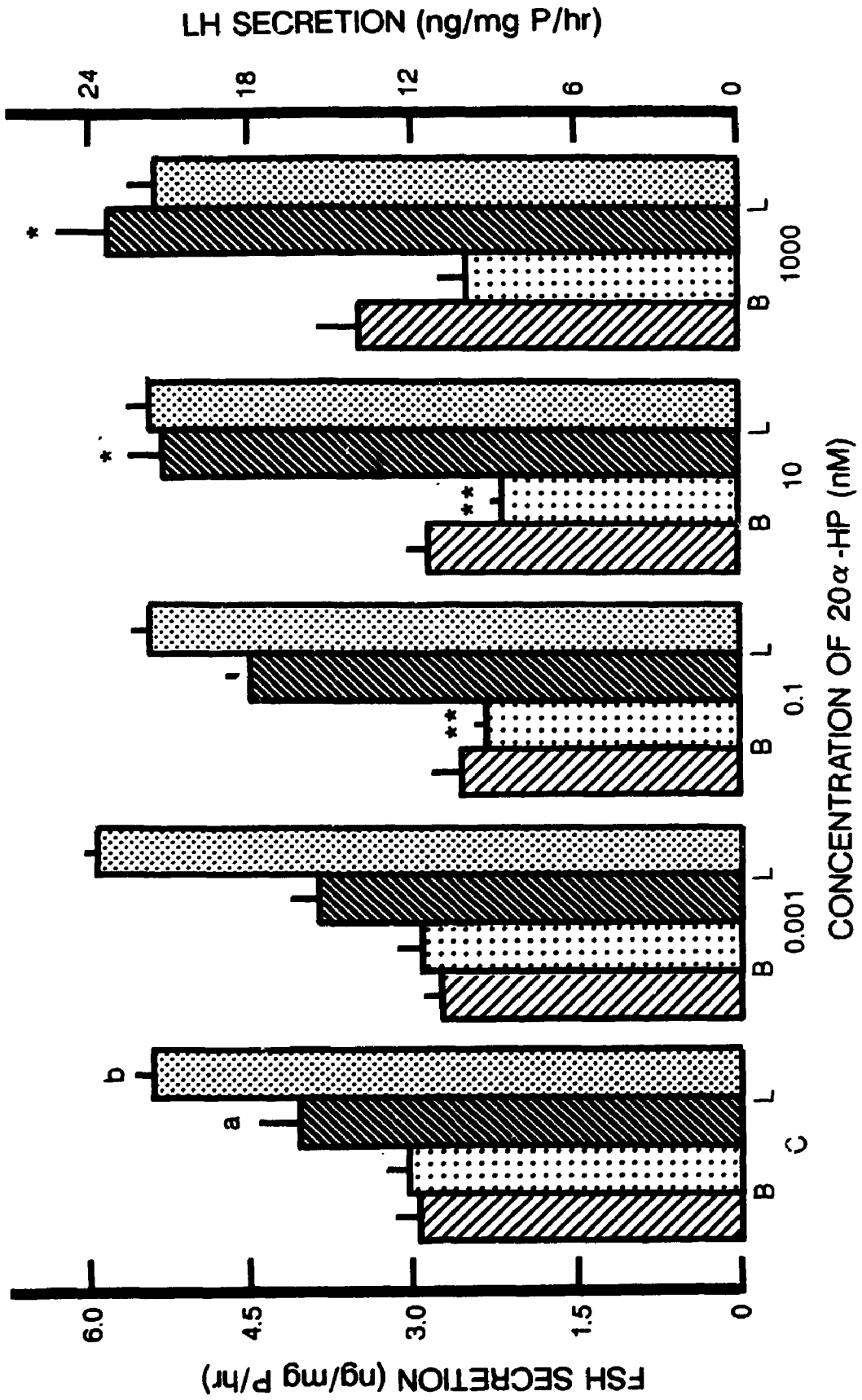

LH SECRETION (ng/mg P/hr)
24
18
12
6
0
FSH SECRETION (ng/mg P/hr)
6.0
4.5
3.0
1.5
0
C
0.001
0.1
10
1000
CONCENTRATION OF 20α-HP (nM)
L
B
a
b
*
**

Table 5: Effect of 17β-estradiol on gonadotropin secretion in cultures of female anterior pituitary cells.

Treatment	Basal		LHRH-Induced	
	FSH	LH	FSH	LH
Control	1.67±0.20	19.65±1.02	2.59±0.15[b]	28.11±2.16[b]
0.001 nM	1.58±0.07	20.91±0.90	2.63±0.22	30.31±2.24
0.1 nM	1.40±0.11	21.96±1.23	2.12±0.17	27.63±1.38
10 nM	1.58±0.05	21.31±1.18	1.90±0.16[a]	25.55±0.98
1000 nM	1.62±0.10	23.15±0.76[a]	2.17±0.13	27.76±1.25

Cultures were derived from mature female rats and were preincubated for 72 hr before treatment. 17β-Estradiol (at doses indicated) and/or LHRH (10 nM) were given and media sampled 24 hr later (see Section 2.4). Results shown are the mean±SEM of n=4-6 replicates and are expressed as ng RP-2/mg total culture protein/hr.

[a] $p<0.05$ compared to respective control
[b] $p<0.01$ compared to basal control.

in Figure 25. 3HP alone selectively inhibited LHRH-induced FSH secretion ($p<0.01$) but had no effect on basal FSH. This may be due to the synergistic stimulating effect of 17β-estradiol and progesterone present in the treatment medium. PFF-I, on the other hand, selectively inhibited basal FSH release by 40% under these experimental conditions ($p<0.05$) but did not differentiate between gonadotropins and suppressed LHRH-induced FSH ($p<0.001$) and LH ($p<0.05$) secretion (Figure 25). Treatment with either 3HP or PFF-I elicited a slight increase in basal LH levels ($p<0.05$), an unusual finding. In cultures treated with both 3HP and PFF-I, basal FSH levels were reduced ($p<0.01$ compared to control) by the same amount as PFF-I alone, while the combination of 3HP and PFF-I inhibited LHRH-induced FSH secretion to 52% of the control level ($p<0.001$; Figure 25). The combined inhibitory effect of 3HP and PFF-I was greater than that produced by either 3HP or PFF-I alone ($p<0.05$).

The possible interaction of 3HP with the gonadal steroids dihydrotestosterone, 17β-estradiol and progesterone in the regulation of in vitro gonadotropin secretion was examined (Table 6). Treatment with 10 nM 3HP resulted in a 33% reduction of basal FSH secretion ($p<0.05$) with no effect on basal LH. LHRH-induced FSH release was also selectively suppressed by 3HP ($p<0.01$). Dihydrotestosterone (10 nM), in the presence of 10 nM 3HP, increased the basal and LHRH-induced secretion of FSH ($p<0.01$). This stimulation of basal FSH release is similar to that seen with dihydrotesoterone alone (Figure 23). When cultures were treated with 10 nM 17β-estradiol and 3HP, both basal FSH ($p<0.01$) and LH ($p<0.05$) secretion were stimulated. Enhancement of basal LH secretion by 17β-estradiol was noted previously (Table 5). Estrogen showed no synergism or additive effect with 3HP in reducing LHRH-induced

Figure 25: Comparison of the effect of 3HP and porcine follicular fluid inhibin (PFF-I) on basal FSH () and LH ( ) secretion and on LHRH-induced FSH ( ) and LH ( ) secretion in primary cultures of adult female rat anterior pituitary cells. Cultures were prepared as described (Section 4.2) and treated as follows. Treatment medium was M199 containing 10 nM estradiol-17β and 1000 nM progesterone to increase basal gonadotropin secretion levels. Treatments were with/without 3HP (10 nM) and/or LHRH (10 nM) and/or PFF-I (300 μM) and cells were incubated a further 24 hr. Medium was collected for analysis of gonadotropins by RIA and assayed in duplicate. Bars represent the mean±SEM of n=5-6 replicates and results are expressed as ng RP-2 secreted/mg total culture protein (P)/hr.

B, basal

L, LHkH-induced

*$p<0.05$, **$p<0.01$ and ***$p<0.001$ compared to respective control

[a]$p<0.05$ and [b]$p<0.001$ compared to basal control

[c]$p<0.05$ compared to 3HP alone

[d]$P<0.05$ compared to PFF-I alone

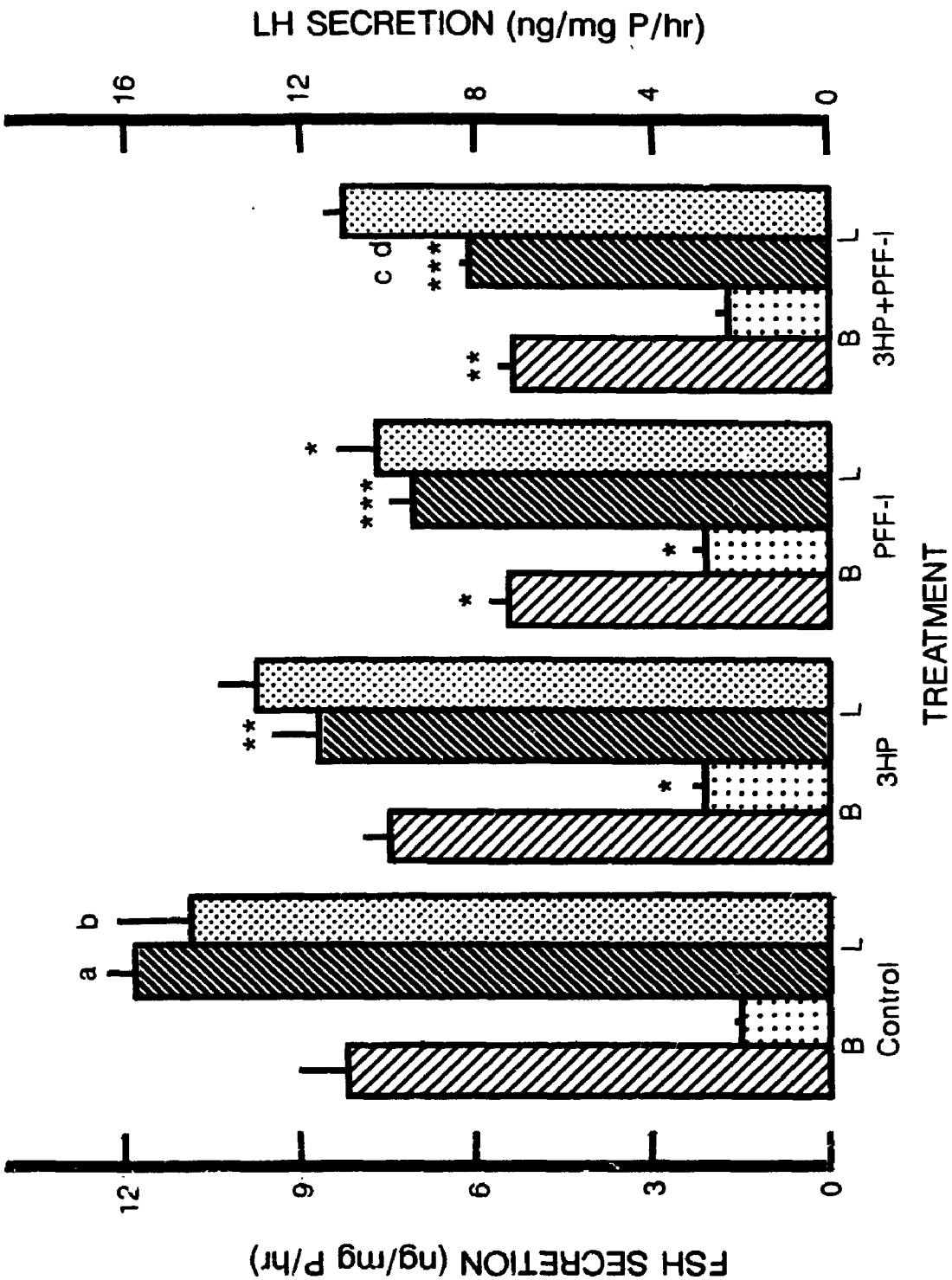

LH SECRETION (ng/mg P/hr)
16
12
8
4
0
c d
***
**
*
***
*
*
**
*
a b
L
B
3HP+PFF-I
PFF-I
3HP
Control
TREATMENT
12
9
6
3
0
FSH SECRETION (ng/mg P/hr)

Table 6: Effect of 3HP and dihydrotestosterone (DHT) or 17β-estradiol (E_2) or progesterone (Prog) on gonadotropin secretion in cultures of female anterior pituitary cells.

Treatment	Basal		LHRH-Induced	
	FSH	LH	FSH	LH
Control	3.21±0.31	4.15±0.24	3.38±0.07	14.34±2.94[d]
3HP	2.16±0.13[a]	3.82±0.26	2.87±0.06[b]	13.11±3.17
3HP+ 1pM DHT	4.00±0.74	4.02±0.30	3.33±0.27	10.96±1.99
3HP+10nM DHT	6.29±0.80[b]	4.64±0.71	5.33±0.47[b]	13.44±3.27
3HP+ 1pM E_2	4.13±0.81	4.34±0.21	3.27±0.14	25.64±3.75
3HP+10nM E_2	7.23±0.89[b]	6.82±1.07[a]	3.66±0.31	18.67±4.22
3HP+100pM Prog	3.08±0.44	4.29±0.31	3.04±0.15	11.95±1.30
3HP+1uM Prog	7.23±0.96[b]	6.40±0.23[c]	3.37±0.47	12.11±1.90

Cultures were derived from mature female rats and preincubated for 72 hr before treatment. 3HP and LHRH were used at 10 nM and media samples taken 24 hr after treatment (see Section 2.4). Results show the mean±SEM of n=4-6 replicates and are expressed as ng RP-2/mg total culture protein/hr.

[a]$p<0.05$, [b]$p<0.01$ and [c]$p<0.001$ compared to respective control
[d]$p<0.05$ compared to basal control

FSH secretion even though both steroids are capable of such an effect (Table 5, Table 6). The combination of 10 nM 3HP with 1 μM progesterone in the culture medium resulted in increases in the basal release of LH ($p<0.001$) and FSH ($p<0.01$) similar to the trend seen with progesterone alone in Figure 20. It therefore appears that the other gonadal steroids have the ability to oppose the action of 3HP *in vitro*.

3.2.3 Structural Isomers of 3HP and Other C_{21} Steroids

It was of interest to study the effects on cultured pituitary cells of various metabolites of 3HP as well as steroids which are structurally related to 3HP, to determine if 3HP is responsible for the FSH-suppressing effect, or if a metabolite is the active form.

The isomer, 3β-hydroxy-4-pregnen-20-one (3βHP), was employed in primary cultures of rat anterior pituitary cells at doses of 0, 0.001, 0.1, 10 and 1000 nM in the presence or absence of 10 nM LHRH and secreted gonadotropin levels examined after 24 hr. 3βHP had no effect on either basal or LHRH-induced FSH secretion (results not shown) but it did affect the secretion of LH. Figure 26 shows that the highest dose of 3βHP (1000 nM) significantly increased basal LH secretion ($p<0.05$) while the same dose inhibited LHRH-induced LH secretion ($p<0.001$) to 42% of the LHRH-control level.

Other pregnenes and one androstene compound were examined in this model culture system for FSH-suppressing activity. 3α,20α-dihydroxy-4-pregnene had no significant effect on *in vitro* gonadotropin secretion (Table 7) but a downward trend in the secretion of basal and LHRH-induced FSH secretion is evident. Another pregnene-diol, 3α,20β-dihydroxy-4-pregnene did not modify FSH secretion in cultured anterior

Figure 26: Effect of 3β-hydroxy-4-pregnen-20-one (3βHP) on basal () and LHRH-induced () LH secretion in primary cultures of adult female rat anterior pituitary cells. Cultures were prepared and treated as described (Section 2.4) and incubated for 24 hr following treatment with 0 (C: Control), 0.001, 0.1, 10 or 1000 nM 3βHP and/or 10 nM LHRH. Medium was collected for analysis of gonadotropins by RIA and assayed in duplicate. Bars represent the mean±SEM of n=5-6 replicates and results are expressed as ng RP-2 secreted/mg total culture protein (P)/hr. Results for FSH are not shown since 3βHP had no effect on FSH secretion.

*$p<0.05$ and ***$p<0.001$ compared to respective control

[a]$p<0.001$ compared to basal control

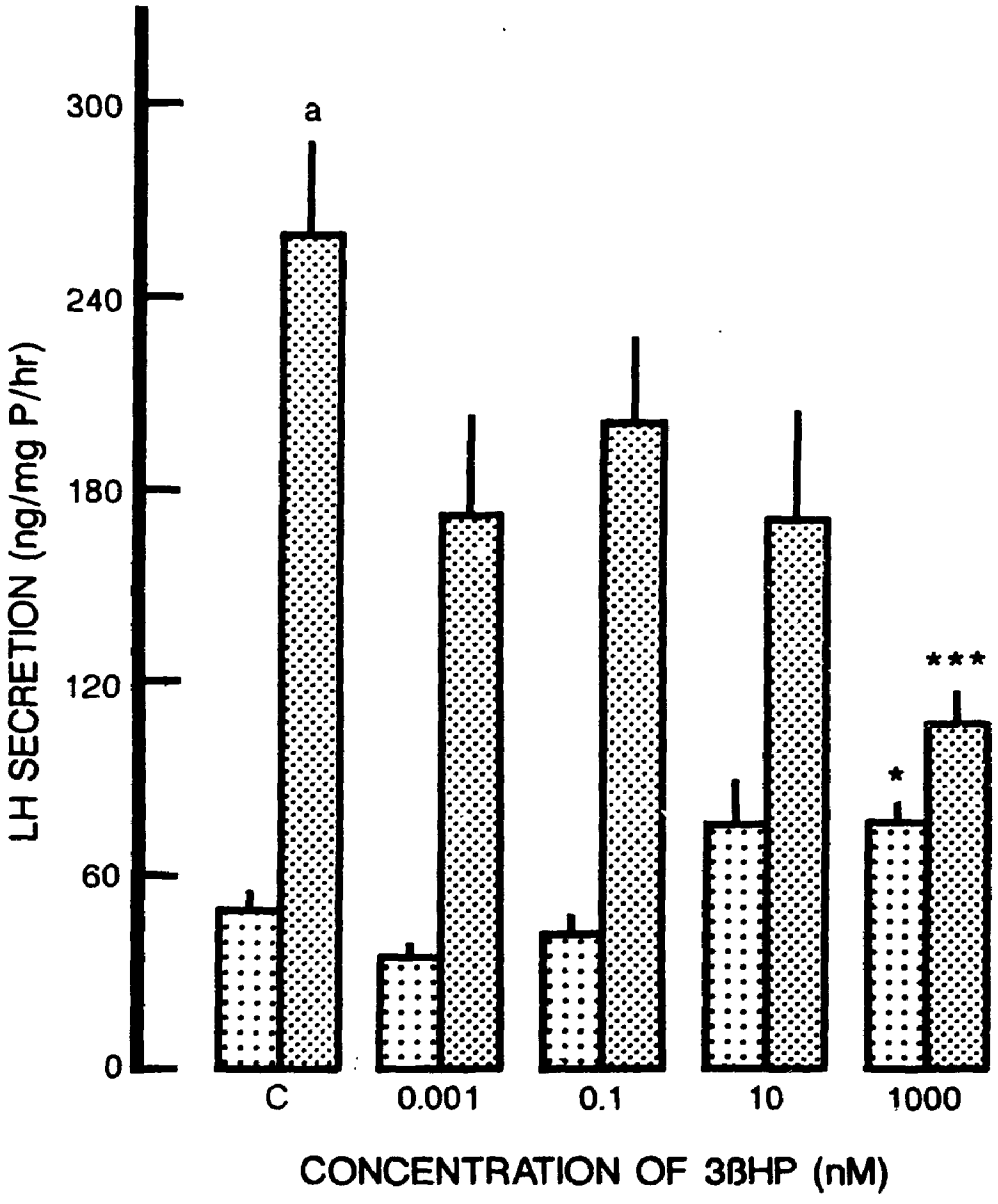

300
240
180
120
60
0
LH SECRETION (ng/mg P/hr)
a
*
***
C
0.001
0.1
10
1000
CONCENTRATION OF 3βHP (nM)

Table 7: Effect of pregnenes and androstene structurally-related to 3HP on gonadotropin secretion in primary cultures of female anterior pituitary cells.

Steroid/Dose	Basal		LHRH-Induced	
	FSH	LH	FSH	LH
3α, 20α-Dihydroxy-4-pregnene				
Control	4.5±0.80	14.3±1.22	10.5±0.45[c]	29.4±1.02[c]
0.001 nM	3.6±0.33	12.3±0.82	10.0±0.54	38.1±5.30
0.1 nM	5.9±1.12	14.3±1.47	9.7±1.61	29.5±1.95
10 nM	3.9±0.28	12.5±0.53	9.0±1.48	35.8±11.2
1000 nM	4.3±0.18	12.3±1.35	8.0±1.16	51.0±13.4
3α, 20β-Dihydroxy-4-pregnene				
Control	3.4±0.07	21.9±1.89	5.3±0.22[c]	43.9±1.89[c]
0.001 nM	3.6±0.41	23.0±0.62	5.6±0.33	32.9±1.43
0.1 nM	3.6±0.18	18.8±1.29	5.1±0.26	39.3±5.88
10 nM	3.3±0.20	18.6±1.58	5.3±0.39	40.7±4.30
1000 nM	3.9±0.21	22.5±1.85	5.5±0.34	60.9±6.35[a]
20β-Hydroxy-4-pregnen-3-one				
Control	1.9±0.14	9.7±0.38	2.8±0.10[c]	19.0±1.38[c]
0.001 nM	1.9±0.16	9.7±0.69	3.3±0.26	24.5±2.32
0.1 nM	1.8±0.11	8.4±0.63	2.4±0.18	20.7±0.88
10 nM	2.0±0.15	12.4±0.86	2.7±0.08	19.6±1.59
1000 nM	2.4±0.17	10.2±0.92	2.8±0.19	17.2±0.95
3α-Hydroxy-4-androsten-17-one				
Control	1.1±0.04	6.5±0.30	2.4±0.23[c]	14.8±0.61[c]
0.001 nM	1.0±0.06	6.2±0.18	1.99±0.18	13.5±0.70
0.1 nM	0.9±0.10	5.9±0.09	2.5±0.27	14.4±0.69
10 nM	1.0±0.10	6.1±0.32	2.3±0.19	15.4±0.59
1000 nM	1.6±0.11[b]	5.8±0.24	3.5±0.33[a]	11.9±0.52[b]

Cultures were derived from mature female rats and preincubated for 72 hr before treatment. Steroids were used at the indicated doses, LHRH employed at 10 nM and media samples taken 24 hr after treatment (see Section 2.4). Results shown the mean±SEM of n=4-6 replicates and are expressed as ng RP-2/mg total culture protein/hr.

[a] $p<0.05$ and [b] $p<0.01$ compared to respective control
[c] $p<0.001$ compared to basal control

pituitary cells, but the highest dose (1000 nM) augmented LHRH-induced LH secretion 1.4-fold ($p<0.05$; Table 7). In vitro release of gonadotropins was not affected by 20β-Hydroxy-4-pregnen-3-one (Table 7). The C_{19} steroid analagous in structure to 3HP, namely 3α-hydroxy-4-androsten-17-one was examined for possible 3HP-like activity. Treatment with 3α-hydroxy-4-androsten-17-one actually increased the basal ($p<0.01$) and LHRH-induced ($p<0.05$) secretion of FSH at a culture concentration of 1000 nM (Table 7). LH secretion after LHRH challenge was decreased by 1000 nM 3α-hydroxy-4-androsten-17-one ($p<0.01$). No effect on the basal release of LH was noted after treatment with 3α-hydroxy-4-androsten-17-one.

The 5α-reduced metabolite of 3HP, 3α-hydroxy-5α-pregnan-20-one, had no effect on the secretion of FSH from primary cultures of rat anterior pituitary cells (results not shown). This particular steroid did, however, stimulate the basal release of LH with the maximum effect occurring at a dose of 0.1 nM ($p<0.001$), where secretion was increased approximately 6-fold over the control (Figure 27). Note that, in this experiment, LH secretion rates are approximately one order of magnitude greater than in all other culture experiments. It is possible that the female rats used as the source of anterior pituitary cells were not random-cycling but were synchronous in a proestrous stage.

Cultures treated with 3β-hydroxy-5β-pregnan-20-one did not exhibit any alteration in FSH secretion (results not shown). Treatment with 3β-hydroxy-5β-pregnan-20-one resulted in a dose-related effect on basal LH secretion which was significantly increased by culture concentrations of 10 ($p<0.05$) and 1000 ($p<0.001$) nM (Figure 28). No significant difference in LH secretion after challenge by LHRH was seen after

Figure 27: Effect of 3α-hydroxy-5α-pregnan-20-one (3α-OH-5α-DHP) on basal (⚄) and LHRH-induced (⚅) LH secretion in primary cultures of adult female rat anterior pituitary cells. Cultures were prepared and treated as described (Section 2.4) and incubated for 24 hr following treatment with 0 (C: Control), 0.001, 0.1, 10 or 1000 nM 3α-hydroxy-5α-pregnan-20-one and/or 10 nM LHRH. Medium was collected for analysis of gonadotropins by RIA and assayed in duplicate. Bars represent the mean±SEM of n=4-6 replicates and results are expressed as ng RP-2 secreted/mg total culture protein (P)/hr. Data on FSH are not shown as 3α-hydroxy-5α-pregnan-20-one did not affect FSH secretion.

*$p<0.05$ and ***$p<0.001$ compared to respective control

[a] $p<0.001$ compared to basal control

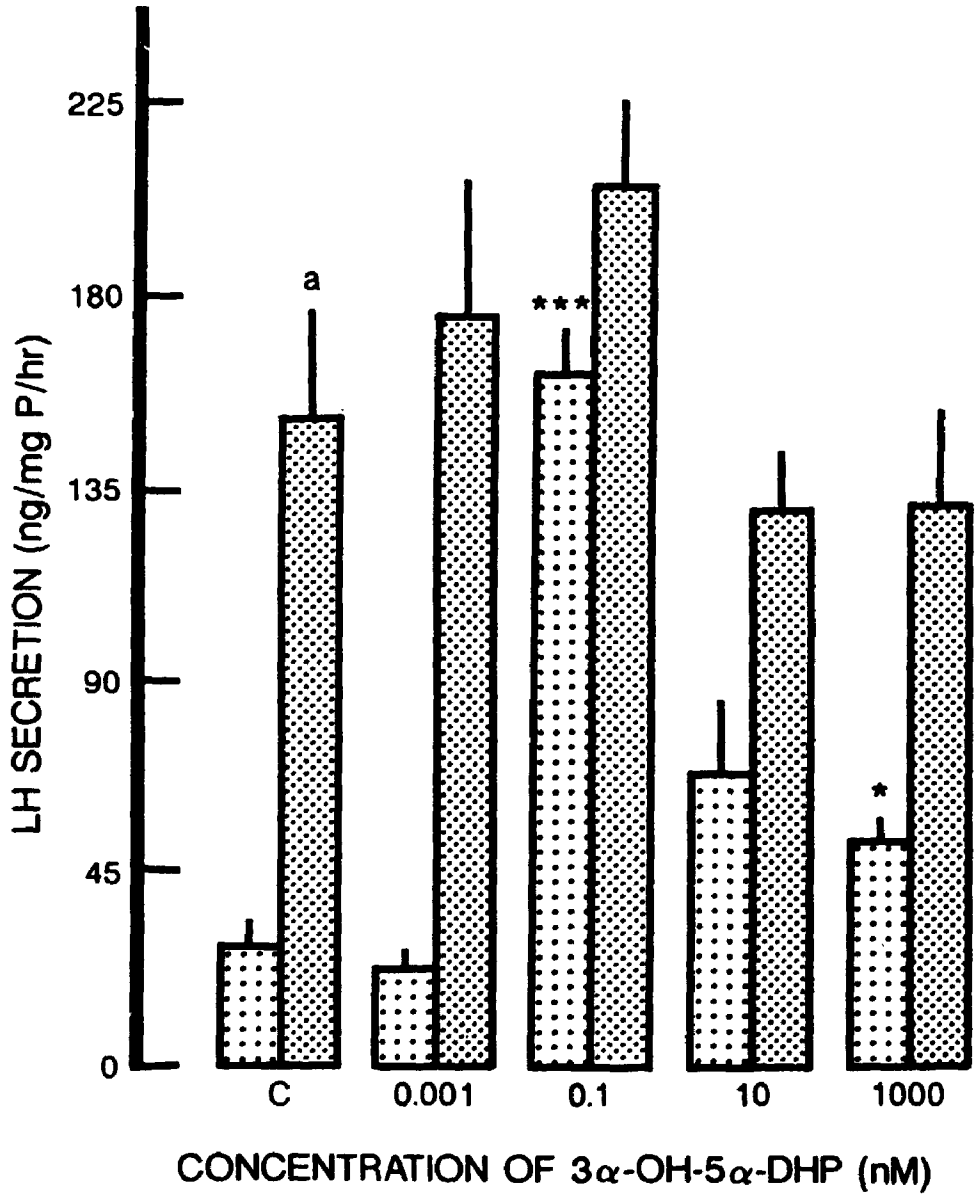
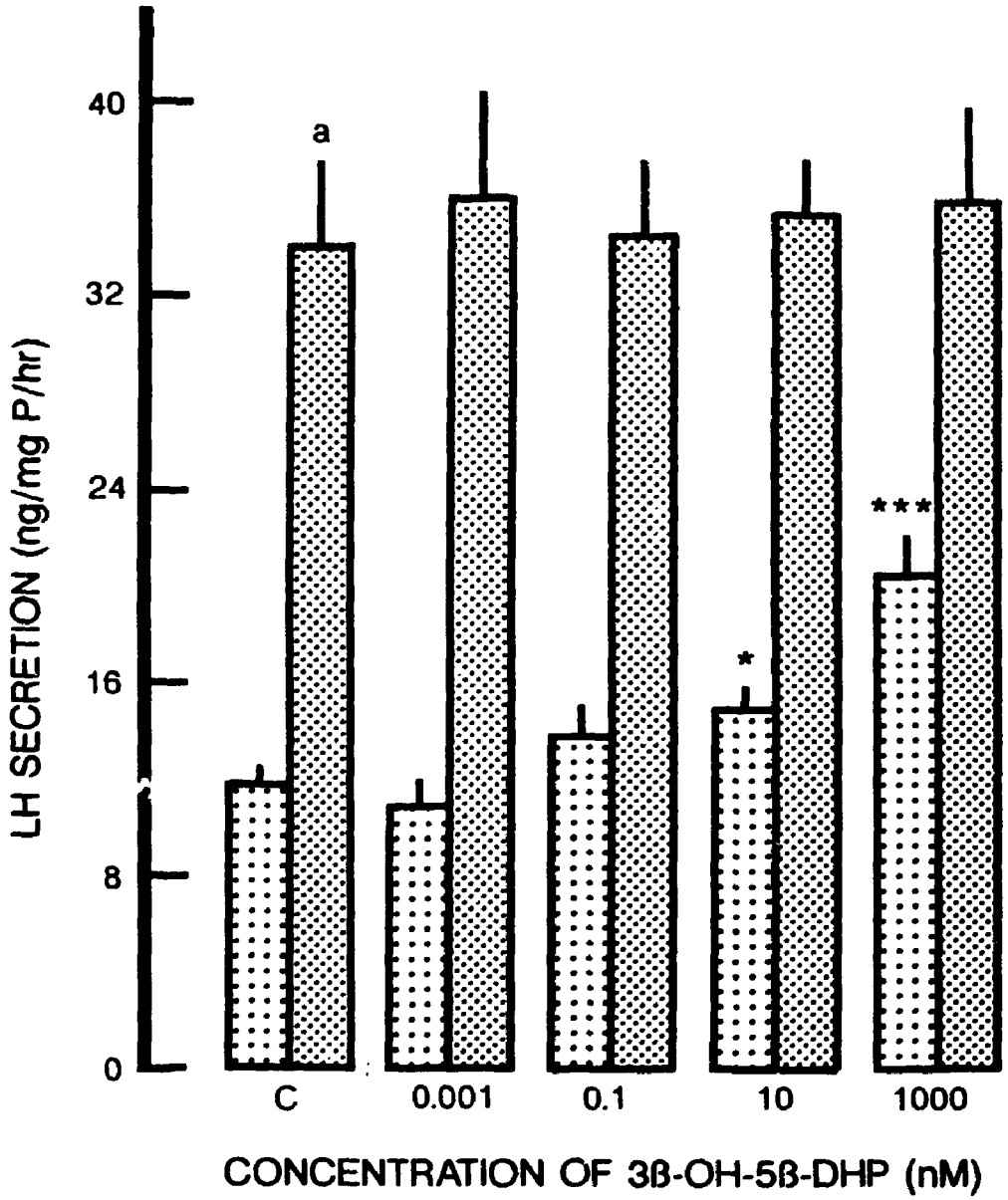

225
180
135
90
45
0
LH SECRETION (ng/mg P/hr)
a
***
*
C
0.001
0.1
10
1000
CONCENTRATION OF 3α-OH-5α-DHP (nM)

Figure 28: Effect of 3β-hydroxy-5β-pregnan-20-one (3β-OH-5β-DHP) on basal (⁘) and LHRH-induced (⁘) LH secretion in primary cultures of adult female rat anterior pituitary cells. Cultures were prepared and treated as described (Section 2.4) and incubated for 24 hr following treatment with 0 (C: Control), 0.001, 0.1, 10 or 1000 nM 3β-hydroxy-5β-pregnan-20-one and/or 10 nM LHRH. Medium was collected for analysis of gonadotropins by RIA and assayed in duplicate. Bars represent the mean±SEM of n=5-6 replicates and results are expressed as ng RP-2 secreted/mg total culture protein (P)/hr. FSH data are not shown since 3β-hydroxy-5β-pregnan-20-one had no effect on FSH secretion.

*$p<0.05$ and ***$p<0.001$ compared to respective control

[a]$p<0.001$ compared to basal control


LH SECRETION (ng/mg P/hr)
40
32
24
16
8
0
a
*
***
C
0.001
0.1
10
1000
CONCENTRATION OF 3β-OH-5β-DHP (nM)

steroid treatment.

The remaining pregnanes examined were employed in cultures of anterior pituitary cells at doses of 0, 0.001, 0.1, 10 and 1000 nM with or without 10 nM LHRH. The secretion of FSH and LH were not influenced by 3β-hydroxy-5α-prenan-20-one (Table 8). However, 5β-pregnane-3,20-dione stimulated the basal secretion of FSH at doses of 0.001 ($p<0.05$), 10 ($p<0.001$) and 1000 nM ($p<0.01$) with maximum stimulation occurring at 10 nM (2.9-fold). No other effect was observed with this steroid. Basal LH secretion was reduced by 28% by a dose of 0.1 nM 3α-hydroxy-5β-pregnan-20-one ($p<0.05$; Table 8).

3.2.4 Studies on the Mechanism of Action of 3HP

Since LHRH stimulates gonadotropin secretion by a mechanism involving the opening of membrane calcium channels (allowing a calcium influx) and the activation of protein kinase C (PKC) (see Section 1.4.1) it was of interest to determine whether 3HP, in its FSH-suppressing activity, interacts with these signal transduction systems in primary cultures of rat anterior pituitary cells.

Cultures of rat anterior pituitary cells were treated with 100 μM A23187 (a calcium ionophore) and/or 10 nM 3HP in the presence or absence of 10 nM LHRH and the medium was sampled for gonadotropins after 24 hr. Treatment with 3HP alone selectively inhibited basal and LHRH-induced FSH secretion ($p<0.05$; Figure 29). A23187 mimicked the effects of LHRH and not only stimulated the basal release of FSH and LH ($p<0.001$), but further stimulated the secretion of FSH after LHRH induction ($p<0.001$; Figure 29). When 3HP and A23187 treatments were combined, basal ($p<0.001$ compared to control) and LHRH-induced FSH secretion was midway between

Table 8: Effect of pregnanes structurally-related to 3HP on gonadotropin secretion in female anterior pituitary cells in culture.

Steroid/Dose	Basal		LHRH-Induced	
	FSH	LH	FSH	LH
3β-Hydroxy-5α-pregnan-20-one				
Control	3.17±0.11	5.85±0.17	4.60±0.42[d]	16.08±0.65[e]
0.001 nM	3.04±0.20	5.80±0.24	3.91±0.42	16.61±0.74
0.1 nM	3.05±0.08	6.22±0.12	4.90±0.24	16.44±0.24
10 nM	3.09±0.12	5.44±0.37	5.20±0.27	16.84±0.43
1000 nM	3.42±0.04	6.43±0.24	5.10±0.37	17.00±0.41
5β-Pregnane-3,20-dione				
Control	0.66±0.04	4.92±0.47	1.70±0.09[e]	21.55±0.54[e]
0.001 nM	1.52±0.33[a]	5.78±0.47	1.93±0.12	20.46±1.75
0.1 nM	1.01±0.29	4.77±0.24	1.67±0.09	21.38±1.36
10 nM	1.91±0.19[c]	4.80±0.36	1.55±0.16	22.89±0.91
1000 nM	1.78±0.28[b]	5.35±0.25	1.96±0.21	22.22±2.11
3α-Hydroxy-5β-pregnan-20-one				
Control	3.54±0.39	14.18±1.29	6.17±0.38[e]	48.89±3.58[e]
0.001 nM	3.32±0.30	11.87±1.07	6.30±0.63	53.20±1.53
0.1 nM	3.08±0.59	10.14±1.12[a]	5.34±0.58	57.51±1.98
10 nM	3.88±0.39	12.49±1.11	4.75±0.30	40.11±4.45
1000 nM	2.43±0.45	12.33±0.93	6.96±0.25	55.91±3.08

Cultures were derived from mature female rats and preincubated for 72 hr before treatment. Steroids were used at the doses indicated, LHRH was used at 10 nM and media were sampled 24 hr after treatment (see Section 2.4). Results show the mean±SEM of n=4-6 replicates and are expressed as ng RP-2/mg total culture protein/hr.

[a]$p<0.05$, [b]$p<0.01$ and [c]$p<0.001$ compared to respective control
[d]$p<0.01$ and [e]$p<0.001$ compared to basal control

Figure 29: Effect of 3HP and the calcium ionophore, A23187, on basal FSH () and LH () secretion and on LHRH-induced FSH () and LH ( ) secretion in primary cultures of adult female rat anterior pituitary cells. Cultures were prepared and treated as described (Section 2.4) and incubated for 24 hr following treatment with/without 10 nM 3HP and/or 100 μM A23187 and/or 10 nM LHRH. Medium was collected for analysis of gonadotropins by RIA and assayed in duplicate. Bars represent the mean±SEM of n=4-6 replicates and results are expressed as ng RP-2 secreted/mg total culture protein (P)/hr.

B, basal

L, LHRH-induced

*p<0.05, **p<0.01 and ***p<0.001 compared to respective control

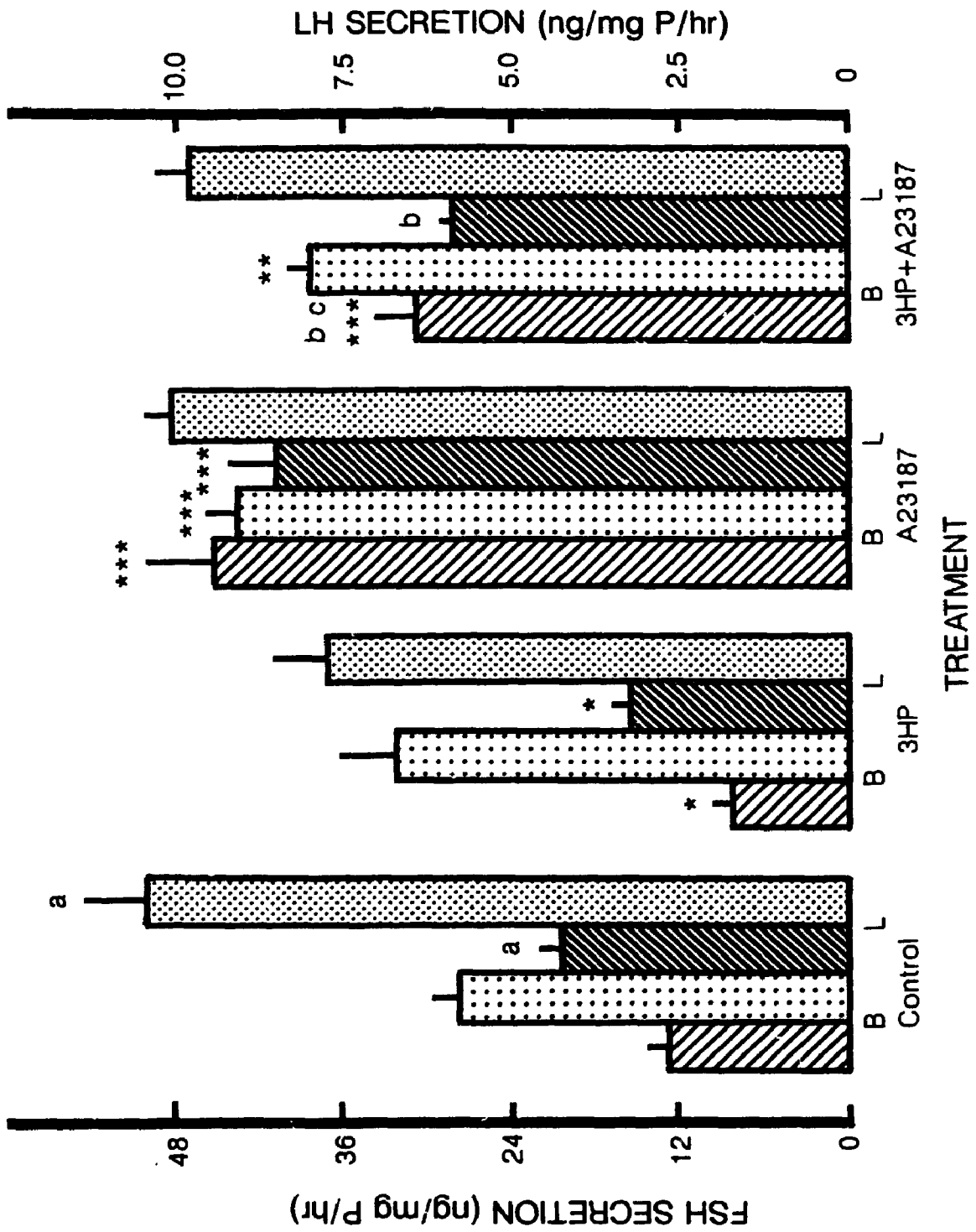
[a]p<0.01 compared to basal control

[b]p<0.05 compared to A23187 alone

[c]p<0.001 compared to 3HP alone

Factorial analysis showed the following main effects and interactions on FSH secretion: 3HP (p<0.001); A23187 (p<0.001); LHRH (p<0.05); LHRH and 3HP (p<0.001); A23187 and LHRH (p<0.01).

Factorial analysis showed the following main effects and interactions on LH secretion: A23187 (p<0.001); LHRH (p<0.001).


LH SECRETION (ng/mg P/hr)
10.0
7.5
5.0
2.5
0
FSH SECRETION (ng/mg P/hr)
48
36
24
12
0
B
L
Control
3HP
A23187
3HP+A23187
TREATMENT
a
b
b c
*
**
***

that seen under conditions of either 3HP or A23187 alone (ie. 3HP opposed the stimulating effect of A23187 or vice versa ($p<0.05$)). 3HP did not oppose the A23187 stimulation of basal LH secretion. Factorial analysis confirmed the main effects of 3HP or A23187 ($p<0.001$) or LHRH ($p<0.05$) on FSH release and showed a significant interaction between A23187 and LHRH ($p<0.01$) and, 3HP and LHRH ($p<0.001$). The main effects of LHRH or A23187 ($p<0.001$) on LH secretion were confirmed and no interactions between treatments were seen with similar analysis.

The dihydropyridine-sensitive calcium channel antagonist, verapamil (100 μM), was employed along with 3HP and LHRH in the same experiment as the calcium ionophore A23187 (the control and 3HP treatments are the same as those above and therefore will not be repeated here). Figure 30 shows that verapamil inhibited both the basal and LHRH-induced secretion of LH ($p<0.01$ and $p<0.001$, respectively). Verapamil stimulated the basal release of FSH ($p<0.01$) and did not reduce LHRH-induced FSH release. When 3HP and verapamil were used together in culture, basal FSH release was again stimulated ($p<0.01$ compared to basal control or 3HP alone) and, although basal LH was somewhat reduced by this treatment, the level of LH in the medium was not significantly different from any of the other treatments. The combined treatment (3HP + verapamil) resulted in inhibition of LHRH-induced LH release ($p<0.001$) that was not different from that produced by verapamil alone. The effect of 3HP and verapamil on LHRH-induced FSH secretion was dramatic and FSH release was reduced to about 7% of the control level ($p<0.001$; Figure 30) which was significantly less than either 3HP or verapamil alone ($p<0.001$). Analysis of these results using the factorial method confirmed the main effects of 3HP or verapamil ($p<0.001$) or LHRH ($p<0.5$) on FSH secretion

Figure 30: Effect of 3HP and the dihydropyridine calcium channel antagonist, Verapamil, on basal FSH (▨) and LH (⁘) secretion and on LHRH-induced FSH (▨) and LH (⁘) secretion in primary cultures of adult female rat anterior pituitary cells. Cultures were prepared and treated as described (Section 2.4) and incubated for 24 hr following treatment with/without 10 nM 3HP and/or 100 μM Verapamil and/or 10 nM LHRH. Medium was collected for analysis of gonadotropins by RIA and assayed in duplicate. Bars represent the mean±SEM of n=4-6 replicates and results are expressed as ng RP-2 secreted/mg total culture protein (P)/hr.

B, basal

L, LHRH-induced

*$p<0.05$, **$p<0.01$ and ***$p<0.001$ compared to respective control

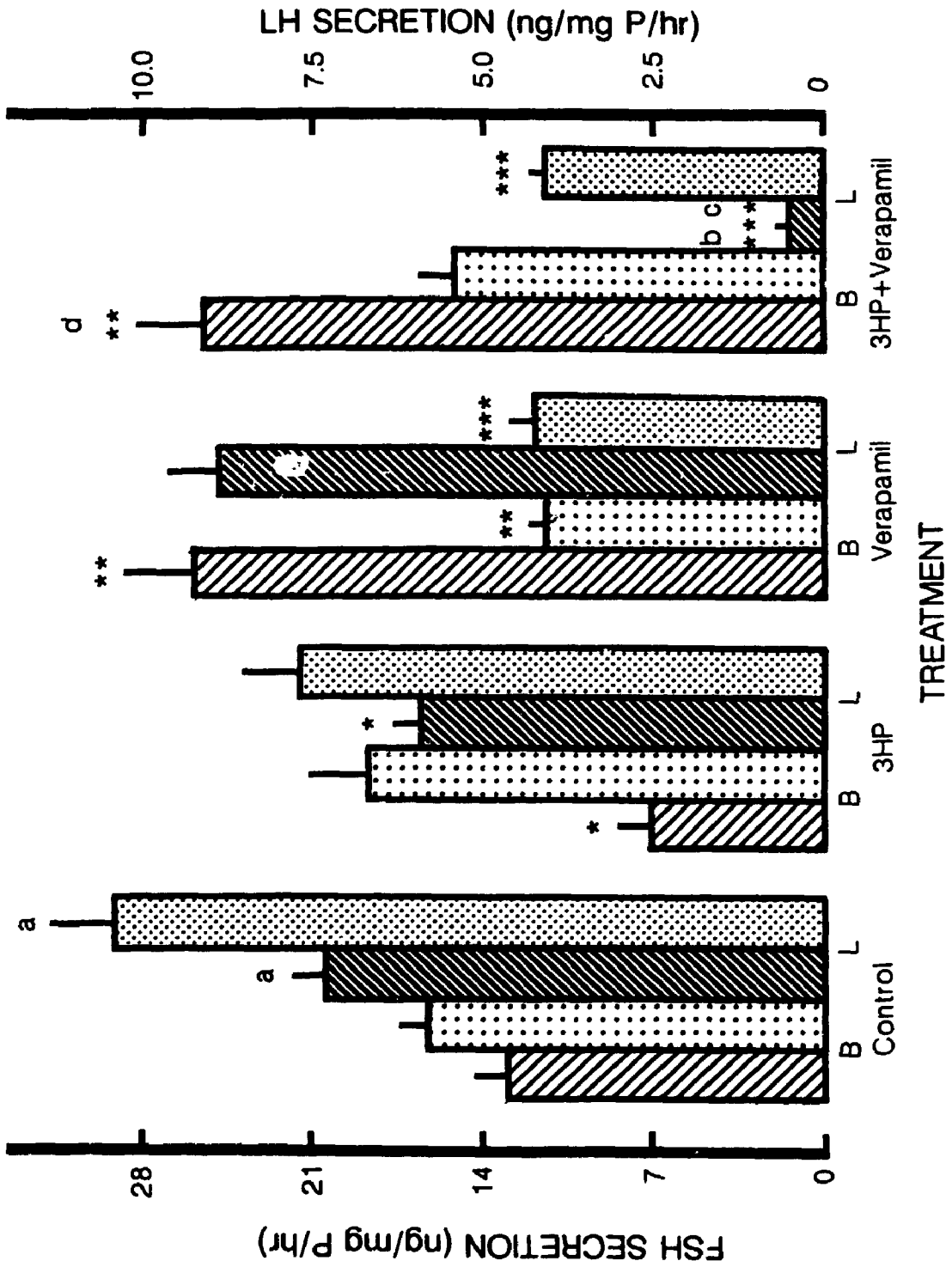
[a]$p<0.01$ compared to basal control

[b]$p<0.001$ compared to Verapamil alone

[c]$p<0.001$ and [d]$p<0.01$ compared to 3HP alone

Factorial analysis showed the following main effects and interactions on FSH secretion: 3HP ($p<0.001$); verapamil ($p<0.001$); LHRH ($p<0.05$); 3HP and verapamil ($p<0.01$); 3HP and LHRH ($p<0.001$); 3HP and verapamil and LHRH ($p<0.001$).

Factorial analysis showed the following main effects and interactions on LH secretion: verapamil ($p<0.001$); LHRH ($p<0.01$); verapamil and LHRH ($p<0.001$).


LH SECRETION (ng/mg P/hr)
10.0
7.5
5.0
2.5
0
FSH SECRETION (ng/mg P/hr)
28
21
14
7
0
B
L
Control
3HP
Verapamil
3HP+Verapamil
TREATMENT
a
a
*
*
**
**
***
**
***
b c
d
**

and showed interactions between verapamil and 3HP ($p<0.01$), LHRH and 3HP ($p<0.001$) and between LHRH, verapamil and 3HP ($p<0.001$) on FSH release. Similar analysis of the LH data confirmed the effect of either verapamil ($p<0.001$) or LHRH ($p<0.01$) on LH secretion and showed a significant interaction between the effects of LHRH and verapamil on LH secretion ($p<0.001$).

Another dihydropyridine-sensitive calcium channel antagonist, nifedipine, was employed in a similar experiment. Nifedipine (100 μM) significantly reduced basal ($p<0.05$) and LHRH-induced ($p<0.001$) secretion of LH (Figure 31). Nifedipine treatment resulted in significant increases in both basal ($p<0.01$) and LHRH-induced ($p<0.05$) FSH release (Figure 31). When 100 μM nifedipine and 10 nM 3HP were combined in a single treatment, LHRH-induced LH secretion was reduced in comparison to the LHRH-treated control ($p<0.05$) but not as markedly as when cultures were treated with nifedipine alone ($p<0.01$). Basal LH secretion was not significantly different from controls after treatment with both 3HP and nifedipine, but was slightly greater than in cultures treated with nifedipine alone ($p<0.05$). Basal FSH release was enhanced over controls in the combined treatment ($p<0.01$) where nifedipine appeared to oppose the FSH-suppressing effect of 3HP ($p<0.01$). In combination, 3HP and nifedipine markedly depressed LHRH-induced FSH release by cultured pituitary cells ($p<0.001$) to about 9% of the control level (Figure 31). This inhibition was significantly greater than that induced by 3HP alone ($p<0.001$) and resulted in FSH levels lower than those seen after incubation with nifedipine alone ($p<0.001$). Again, analysis by factorials confirmed the main effects of 3HP or nifedipine ($p<0.001$) or LHRH ($p<0.05$) on FSH release and indicated that there is a

Figure 31: Effect of 3HP and the dihydropyridine calcium channel antagonist, Nifedipine, on basal FSH () and LH () secretion and on LHRH-induced FSH () and LH ( ) secretion in primary cultures of adult female rat anterior ppituitary cells. Cultures were prepared and treated as described (Section 2.4) and incubated for 24 hr following treatment with/without 10 nM 3HP and/or 100 μM Nifedipine and/or 10 nM LHRH. Medium was collected for analysis of gonadotropins by RIA and assayed in duplicated. Bars represent the mean±SEM of n=4-6 replicates and results are expressed as ng RP-2 secreted/mg total culture protein (P)/hr.

B, basal

L, LHRH-induced

*$p<0.05$, **$p<0.01$ and ***$p<0.001$ compared to respective control

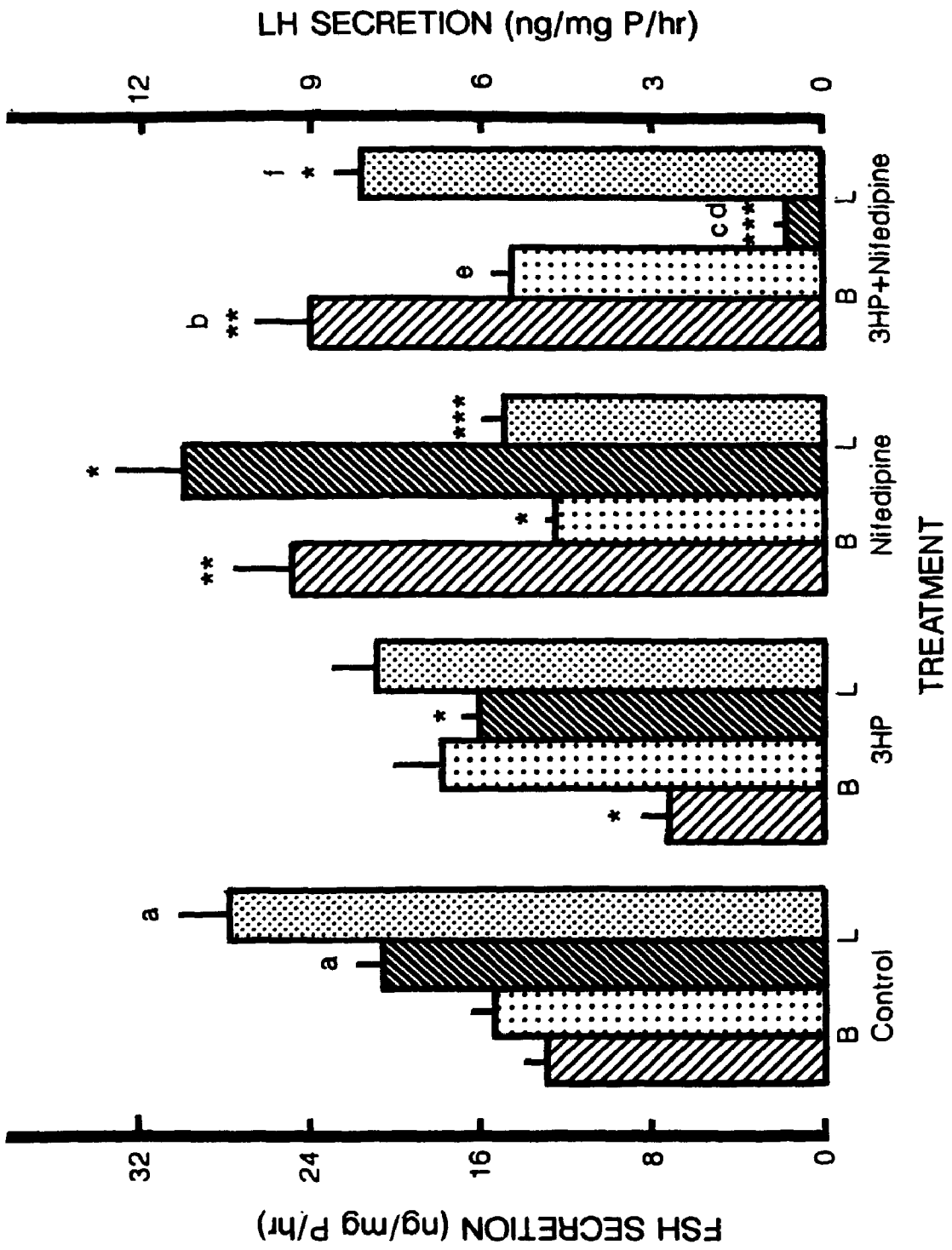
[a]$p<0.01$ compared to basal control

[b]$p<0.01$ and [c]$p<0.001$ compared to 3HP alone

[d]$p<0.001$, [e]$p<0.05$ and [f]$p<0.01$ compared to Nifedipine alone

Factorial analysis showed the following main effects and interactions on FSH secretion: 3HP ($p<0.001$); nifedipine ($p<0.001$); LHRH ($p<0.05$); 3HP and nifedipine ($p<0.01$); 3HP and LHRH ($p<0.001$); LHRH and nifedipine ($p<0.001$); 3HP and LHRH and nifedipine ($p<0.001$).

Factorial analysis showed the following main effects and interactions on LH secretion: nifedipine ($p<0.001$); LHRH ($p<0.001$); 3HP and nifedipine ($p<0.01$); 3HP and nifedipine and LHRH ($p<0.01$).


LH SECRETION (ng/mg P/hr)
12
9
6
3
0
f
*
cd
***
e
b
**
B
L
3HP+Nifedipine
***
*
*
**
Nifedipine
*
*
3HP
a
a
Control
TREATMENT
32
24
16
8
0
FSH SECRETION (ng/mg P/hr)

significant interaction between 3HP and nifedipine, 3HP and LHRH, nifedipine and LHRH and, between all 3 treatments ($p<0.001$). The main effects of nifedipine and LHRH ($p<0.001$) on LH secretion were upheld by factorial analysis which also showed an interaction between 3HP and nifedipine or 3HP and nifedipine and LHRH ($p<0.01$). These interactions were not seen with t-test.

In a separate experiment, an attempt was made to specifically activate or inhibit protein kinase C (PKC) in cultures of anterior pituitary cells treated with 10 nM 3HP, with or without 10 nM LHRH. Figure 32 (and Figure 33) show that 3HP selectively inhibited basal ($p<0.01$) and LHRH-induced ($p<0.001$) FSH secretion by cultured pituitary cells without affecting LH secretion. LHRH stimulated the secretion of both gonadotropins ($p<0.001$).

The specific PKC inhibitor, 1-(5-isoquinolinesulfonly)-2-methylpiperazine dihydrochloride (H-7; Kawamoto and Hidaka, 1984; Ho et al, 1988; Ito et al, 1988), when used at a concentration of 50 μM, slightly elevated the basal secretion of FSH ($p<0.05$). and had no effect on basal LH secretion by pituitary cells in culture (Figure 32). H-7 inhibited LHRH induction of gonadotropin secretion ($p<0.001$). When combined in a single treatment, 3HP and H-7 suppressed LHRH-induced FSH secretion ($p<0.001$) to a level lower than that produced by H-7 alone ($p<0.05$) but not significantly different from 3HP alone (Figure 32). LHRH-induced LH secretion was also reduced by combined (3HP + H-7) treatment ($p<0.001$) but this reduction was similar in magnitude to that achieved by H-7 alone and the level of LH in the medium was much less than that when 3HP alone was employed ($p<0.001$). Analysis of factorials confirmed the main effects of 3HP or LHRH ($p<0.001$) or H-7 ($p<0.05$) on

Figure 32: Effect of 3HP and the protein kinase C inhibitor, H-7, on basal FSH (▨) and LH (⊡) secretion and on LHRH-induced FSH (▨) aud LH (⊡) secretion in primary cultures of adult female rat anterior pituitary cells. Cultures were prepared and treated as described (Section 2.4) and incubated for 24 hr following treatment with/withhout 10 nM 3HP and/or 50 μM H-7 and/or 10 nM LHRH. Medium was collected for analysis of gonadotropins by RIA and assayed in duplicate. Bars represent the mean±SEM of n=5-6 replicates and results are expressed as ng RP-2 secreted/mg total culture protein (P)/hr.

B, basal

L, LHRH-induced

*p<0.05, **p<0.01 and ***p<0.001 compared to respective control

[a]p<0.001 compared tc basal control

[b]p<0.05 compared to 3HP alone

[c]p<0.05 compared to H-7 alone

[d]p<0.001 compared to 3HP alone

Factorial analysis showed the following main effects and interactions on FSH secretion: 3HP (p<0.001); H-7 (p<0.05); LHRH (p<0.001); 3HP and LHRH (p<0.01); 3HP and H-7 (p<0.01); H-7 and LHRH (p<0.01); 3HP and H-7 and LHRH (p<0.01).

Factorial analysis showed the following main effects and interactions on LH secretion: H-7 (p<0.001); LHRH (p<0.001); H-7 and LHRH (p<0.001).

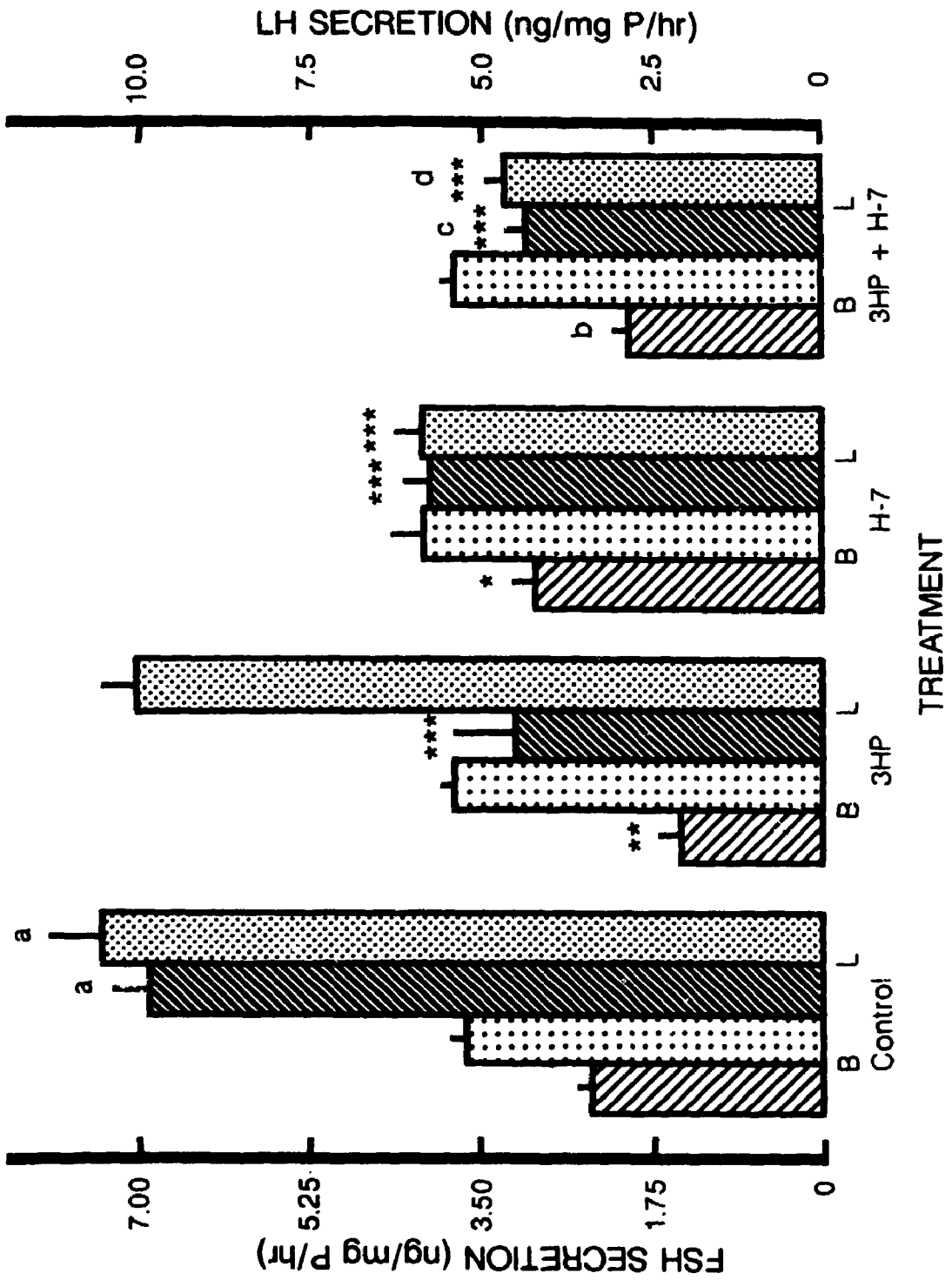

LH SECRETION (ng/mg P/hr)
10.0
7.5
5.0
2.5
0
FSH SECRETION (ng/mg P/hr)
7.00
5.25
3.50
1.75
0
a
a
B
L
Control
**
***
B
L
3HP
*
***
***
B
L
H-7
b
c
d
***
***
B
L
3HP + H-7
TREATMENT

Figure 33: Effect of 3HP and the specific protein kinase C activator, SC-9, on basal FSH () and LH ( ) secretion and on LHRH-induced FSH ( ) and LH ( ) secretion in primary cultures of adult female rat anterior pituitary cells. Cultures were prepared and treated as described (Section 2.4) and incubated for 24 hr following treatment with/without 10 nM 3HP and/or 100 μM SC-9 and/or 10 nM LHRH. Medium was collected for analysis of gonadotropins by RIA and assayed in duplicate. Bars represent the mean±SEM of n=5-6 replicates and results are exppessed as ng RP-2 secreted/mg total culture protein (P)/hr.

B, basal

L, LHRH-induced

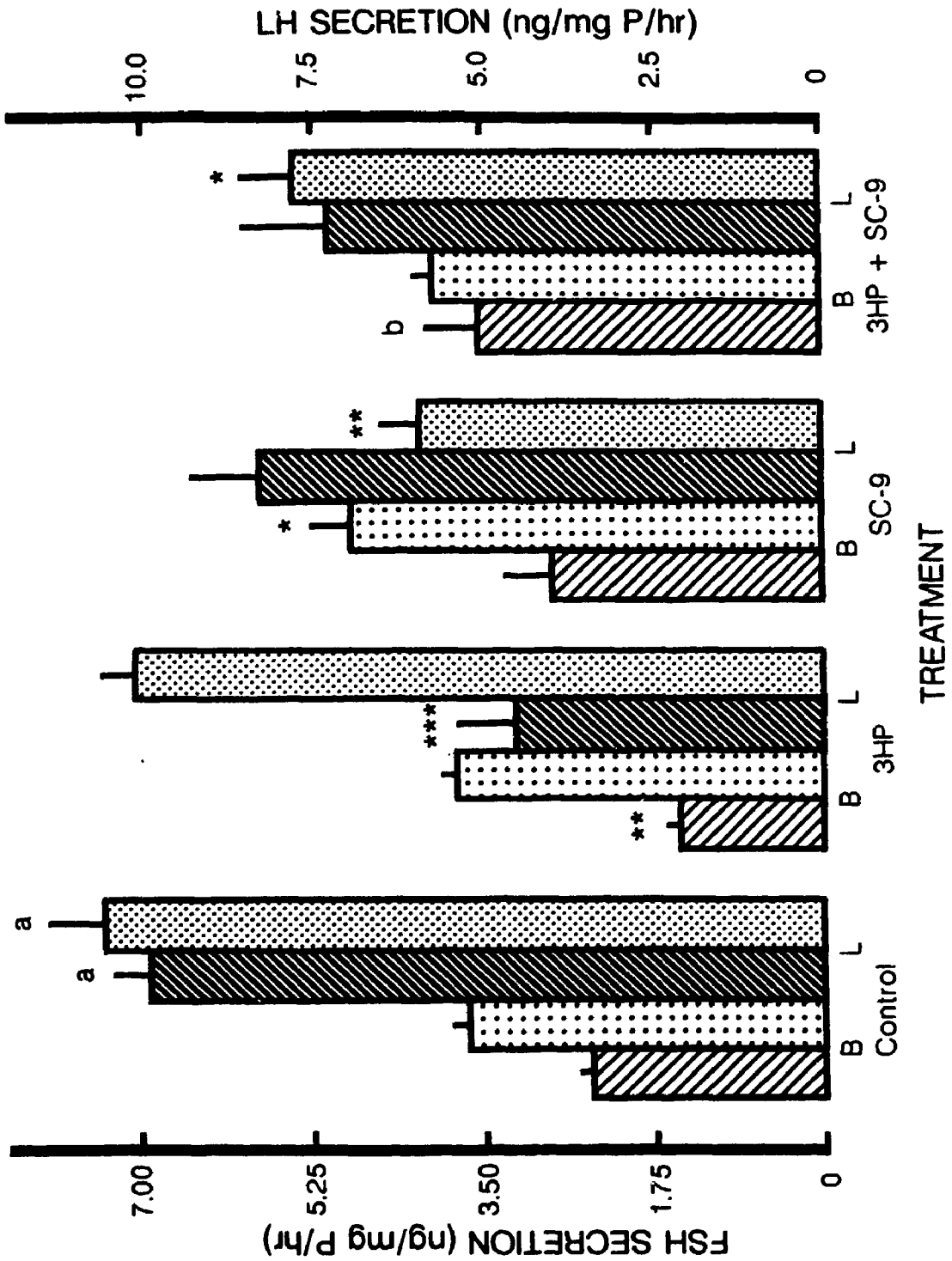
*p<0.05, **p<0.01 and ***p<0.001 compared to respective control

[a]p<0.001 compared to basal control

[b]p<0.01 compared to 3HP alone

Factorial analysis showed the following main effects and interactions on FSH secretion: 3HP (p<0.001); SC-9 (p<0.05); LHRH (p<0.001); 3HP and LHRH (p<0.001); 3HP and SC-9 (p<0.01).

Factorial analysis showed the following main effects and interactions on LH secretion: SC-9 (p<0.01): LHRH (p<0.001); SC-9 and LHRH (p<0.001); SC-9 and LHRH and 3HP (p<0.01).


LH SECRETION (ng/mg P/hr)
10.0
7.5
5.0
2.5
0
FSH SECRETION (ng/mg P/hr)
7.00
5.25
3.50
1.75
0
a
a
b
*
***
**
**
*
Control
3HP
SC-9
3HP + SC-9
B
L
TREATMENT

FSH secretion and showed that there was indeed a significant interaction between 3HP and H-7, and 3HP and H-7 and LHRH ($p<0.001$) as well as the expected interaction between 3HP and LHRH ($p<0.001$). Factorial analysis confirmed the t-test results with respect to LH secretion, H-7 or LHRH have significant effects individually ($p<0.001$) and interact together ($p<0.001$).

An attempt was made to activate PKC in cultured pituitary cells with 100 μM N-(phenylhexyl)-5-chloro-1-naphthalenesulfonamide (SC-9; Ito et al, 1986; Nishino et al, 1986). Gonadotropes did not respond to activation of PKC by SC-9 with any elevation of FSH secretion at either the basal or LHRH-induced level (Figure 33). Activation of PKC by SC-9 stimulated the basal release of LH ($p<0.05$) and inhibited LHRH-induced LH secretion ($p<0.01$). This inhibition of LHRH-induced LH secretion by SC-9 may be due to some sort of down-regulatory phenomenon. The FSH-suppressing action of 3HP was opposed by SC-9 when both 3HP and SC-9 were employed together: basal FSH secretion was not significantly different from the control and was significantly greater than the level seen when 3HP alone was used ($p<0.01$); LHRH-induced FSH secretion also was not significantly different from control (Figure 33). When used together, SC-9 and 3HP significantly reduced LHRH-induced LH secretion ($p<0.05$) but this result was not significantly different from the effect of SC-9 alone. Analysis of factorials confirmed the results obtained with t-test in this experiment: 1) 3HP ($p<0.01$) or SC-9 ($p<0.05$; not shown with t-test) or LHRH ($p<0.001$) significantly affect FSH secretion; 2) 3HP and LHRH and, 3HP and SC-9 interact to affect FSH secretion ($p<0.01$); 3) SC-9 ($p<0.01$) or LHRH ($p<0.001$) affect LH secretion and 4) SC-9 and LHRH interact to affect LH secretion ($p<0.001$).

DISCUSSION

Evidence has accumulated since 1932 to suggest that a seminiferous tubular factor is involved in the regulation of pituitary FSH secretion. McCullagh (1932) argued that organic extracts of testis restore only the androgen-dependent secondary sex characters in castrated rats while aqueous extracts prevent the associated pituitary hypertrophy and therefore, the testis has dual endocrine activity. Lacy and Pettitt (1970) suggested that since Sertoli cells have steroidogenic activity which is under the control of FSH, perhaps the products of Sertoli cell metabolism play a role in the negative feedback regulation of FSH. In a study of men with complete azoospermia it was determined that the Sertoli cell or early germinal elements were responsible for inhibiting FSH secretion since these men had four-fold higher levels of FSH (and no difference in LH) than normal men (VanThiel et al, 1972). FSH levels did not rise in germ cell only-depleted rats, which again suggests the involvement of a Sertoli cell factor in FSH regulation (Krueger et al, 1974). It is now well-established that at least one product of Sertoli cell metabolism, namely inhibin, is capable of regulating FSH secretion both in vivo and in vitro (Steinberger and Steinberger, 1976; Massicotte et al, 1984a; Thomas and Nikitovich-Winer, 1984) but it has also been argued that inhibin alone cannot fully account for FSH inhibition (Decker et al, 1981; Sheth and Vijayalakshmi, 1981) and that inhibin may be a significant regulator of FSH secretion only in the prepubertal male (Summerville and Schwartz, 1981; Hermans et al, 1980; Ultee-vanGessel and deJong, 1987). The evidence presented here indicates that the Sertoli cell steroid, 3α-hydroxy-4-pregnen-20-one (3HP) selectively suppresses FSH secretion both in vivo and in vitro and may be an

integral part of the rat FSH regulatory system.

4.1 3HP SELECTIVELY SUPPRESSES FSH SECRETION IN VIVO

The evidence presented herein shows definitively that 3HP selectively suppresses FSH secretion in the intact and castrate prepubertal male, ovariectomized prepubertal female and castrate adult male rat.

The sensitivity of the male pituitary to 3HP is in place by 10 days of age, the age at which Sertoli cell production of 3HP starts to rise (Wiebe, 1982). At this age the male pituitary is already sensitive to other gonadal steroids (McEwen, 1981; MacLusky and Naftolin, 1981) and inhibin (Ultee-VanGessel and deJong, 1987). Inhibin production by Sertoli cells is also greatest at this time (Rivier et al, 1988). Notably, the greatest suppression of FSH was seen in the gonadectomized adult male, suggesting that pituitary sensitivity to 3HP is more fully developed in the adult as is true for the other gonadal steroids (MacLusky and Naftolin, 1981). It may also be that, in the adult castrate rat, the post-castration rise in serum gonadotropins has not yet reached a plateau and that 3HP is preventing the rise in serum FSH. Some suppression of FSH was also seen in the 55 day old intact male although variability in the data precluded statistical significance. The additional complication of testicular factors present in the adult intact rat may have influenced or modified the effect of 3HP.

The suppression of FSH secretion by 3HP is a unique (among steroids) biological property. An examination of the relevant literature shows that the gonadal steroids, testosterone, dihydrotestosterone and 17β-estradiol suppress both gonadotropins in the castrate male rat but

that the inhibition of LH invariably exceeds that of FSH (see Table 1). This is consistent with the results obtained for dihydrotestosterone, 17β-estradiol and 17α-hydroxyprogesterone in this study where only the highest dose showed inhibition of FSH along with LH.

Progesterone has no effect in the gonadectomized male rat while the glucocorticoids actually increase FSH secretion in either intact or GX rats (Table 1). Although 3HP can be metabolized in vivo to 3α-hydroxy-5α-pregnan-20-one which is reported to increase serum LH (Murphy and Mahesh, 1984b), 3α-hydroxy-5α-pregnan-20-one did not increase serum LH in the intact male rat in this study. This suggests that the increase in serum LH in the intact male after treatment with 3HP was not due to its metabolism to 3α-hydroxy-5α-pregnan-20-one. The LH increasing effect of 3HP was observed on only a few occassions and may be spurious result due to natural variation among the animals. Previous studies used the ovariectomized prepubertal female rat primed with 17β-estradiol and before treatment with 3α-hydroxy-5α-pregnan-20-one, therefore the procedures are not entirely analogous. 5α-pregnane-3,20-dione also increased serum LH in the intact male, a finding not previously reported (Swerdloff et al, 1973).

The activity of 3HP appears to be related to the 3α,4-keto structure of the molecule, since neither the 5α-reduced 3HP, 3α-hydroxy-5α-pregnan-20-one, nor the 3β epimer of 3HP, 3β-hydroxy-4-pregnen-20-one, resulted in decreased FSH secretion. In fact, 3β-hydroxy-4-pregnen-20-one increased serum FSH in the intact male, further suggesting that the 3α-allylic structure of 3HP is important in its action on gonadotropin release from the pituitary.

A hormone must act in a dose-dependent fashion to qualify as a true

physiological regulator. Figure 11 shows that 3α-hydroxy-4-pregnen-20-one-3-acetyl (3HPA) acts in a dose-related manner in castrate rats. 3HPA was also shown to act dose-dependently in intact male rats and not to have an androgenic (stimulation of accesory sex structures) effect (Wiebe and Wood, 1987). 3HPA suppression of FSH occurs at doses as low as 0.5 ug/100 g BW which is considered physiological for most steroids (Gay and Dever, 1971; Eldridge et al, 1974; Dohler and Wuttke, 1975) and is much lower than the dose of testosterone, dihydrotestosterone or 17β-estradiol required to inhibit FSH secretion. It should be noted that until 3HP has been detected in serum, the 3HP effect under discussion may be pharmacological in nature. That is, 3HP may be exerting its FSH-suppressing effect at a site other than a specific 3HP receptor, although this is unlikely in view of the unique properties of 3HP compared to closely related steroids.

In all of the in vivo studies, 3HP selectively reduced FSH secretion with the lowest dose (20 ug/100 g BW) having maximum effect. This finding was somewhat unusual and the switch to the more stable 3-acetyl derivative (3HPA) and the regular use of a lower dose range resulted in consistent, dose-related responses.

4.2 3HP SELECTIVELY SUPPRESSES FSH SECRETION IN VITRO

Primary cultures of rat anterior pituitary cells have been frequently employed to study the influence of gonadal hormones on pituitary gonadotropin secretion. This study shows that 3HP inhibits both the basal and LHRH-induced secretion of FSH in a dose-dependent manner from cultured male and female anterior pituitary cells, with no apparent effect on LH release (Section 3.2.1). Extremely low doses of

3HP (10^{-16} M) appear to have an FSH-suppressing action. Although this dose gives the availability of about 0.2 molecules 3HP per cell, it could be that 3HP mainly affects FSH gonadotropes (approximately 11% of total population; Childs *et al*, 1981), thus increasing the overall availability of the steroid to FSH-secreting cells.

4.2.1 Comparison With Other Gonadal Steroids

Previous studies have shown that the gonadal steroids testosterone, dihydrotestosterone, 17β-estradiol and progesterone increase the basal release of FSH *in vitro* (Table 2). These findings are substantiated by the results presented herein (Figures 20, 22, 23 and Table 5). Dihydrotestosterone, 17β-estradiol and progesterone, at high doses, are all capable of stimulating FSH secretion in the presence of 3HP but 3HP antagonizes (or cancels out) the stimulatory effect of these steroids when used at lower doses (Table 6).

Glucocorticoids are also thought to increase basal FSH secretion (Table 2), while other C_{21} steroids are thought to be inhibitory to FSH secretion *in vitro*. Reports suggest that 20α-hydroxy-4-pregnen-3-one decreases basal FSH secretion in cultured female pituitary cells (Tang and Spies, 1975) but the present study does not support this finding (Figure 24). Progesterone decreases the basal synthesis and secretion of FSH in ovine pituitary cell cultures (Batra *et al*, 1986). It is quite interesting to discover from the literature and the results in this study that, with respect to steroid hormones, only C_{21} steroids have been shown to selectively affect (generally stimulate) basal FSH release *in vitro* and that 3HP is also a C_{21} steroid. Only 3HP is selectively inhibitory to basal FSH secretion *in vitro* (Figures 16, 17 and 18).

Induction of gonadotropin secretion with LHRH is a commonly used tool in the elucidation of gonadal hormone effects on pituitary secretion. Several reports indicate that, in vitro, testosterone, dihydrotestosterone, 17β-estradiol and progesterone all increase the LHRH-induced secretion of FSH while the androgens described decrease, and 17β-estradiol increases, the LHRH-induced secretion of LH (Table 2). However, one report suggests that dihydrotestosterone and testosterone suppress LHRH-induced FSH and LH secretion in cultured pituitary cells (Table 2). The present study has shown a trend similar to the concensus with respect to gonadal steroids: dihydrotestosterone and testosterone do indeed stimulate LHRH-induced FSH secretion (Figures 22 and 23) although no significant effect on LH secretion was noted. The gonadal steroids 5α-pregnane-3,20-dione and 20α-hydroxy-4-pregnen-3-one also appear to affect gonadotropin secretion in vitro (Figures 21 and 24) but in a non-selective manner. In contrast to all these steroids, 3HP differentially inhibits the secretion of FSH after LHRH-stimulation in primary cultures of rat anterior pituitary cells (Figures 16, 17, 18 and 19). This study has also shown that 3HP depresses the synthesis of FSH along with its effect on secretion (Table 4).

4.2.2 3HP and Inhibin Effects In Vitro

Considerable attention has been focussed on inhibin (a Sertoli cell polypeptide) which appears to play a role in the modulation of pituitary FSH secretion. However, inhibin does not modulate the pituitary response to LHRH in vivo consistently (Morris and Asmatullah, 1982) and, although inhibin has been shown to selectively inhibit basal FSH secretion in vitro, it is unanimously agreed that inhibin suppresses the LHRH-

stimulated release of both FSH and LH (Table 2). Bovine inhibin appears to depress basal LH secretion as well (Farnworth et al, 1988). Porcine follicular fluid inhibin (PFF-I), used in this study, was incapable of selectively suppressing basal FSH secretion unless gonadotropes were first stimulated with progesterone and 17β-estradiol to increase gonadotropin secretion (Figure 25). 3HP did not decrease basal FSH secretion in this system, possibly due to the opposing effects of progesterone and 17β-estradiol, but did selectively inhibit LHRH-induced FSH. PFF-I was not capable of a similar selective FSH reduction under both basal and LHRH-stimulated conditions. Therefore, it appears that only 3HP is capable of a consistent selective reduction of both basal and LHRH-induced FSH secretion in vitro.

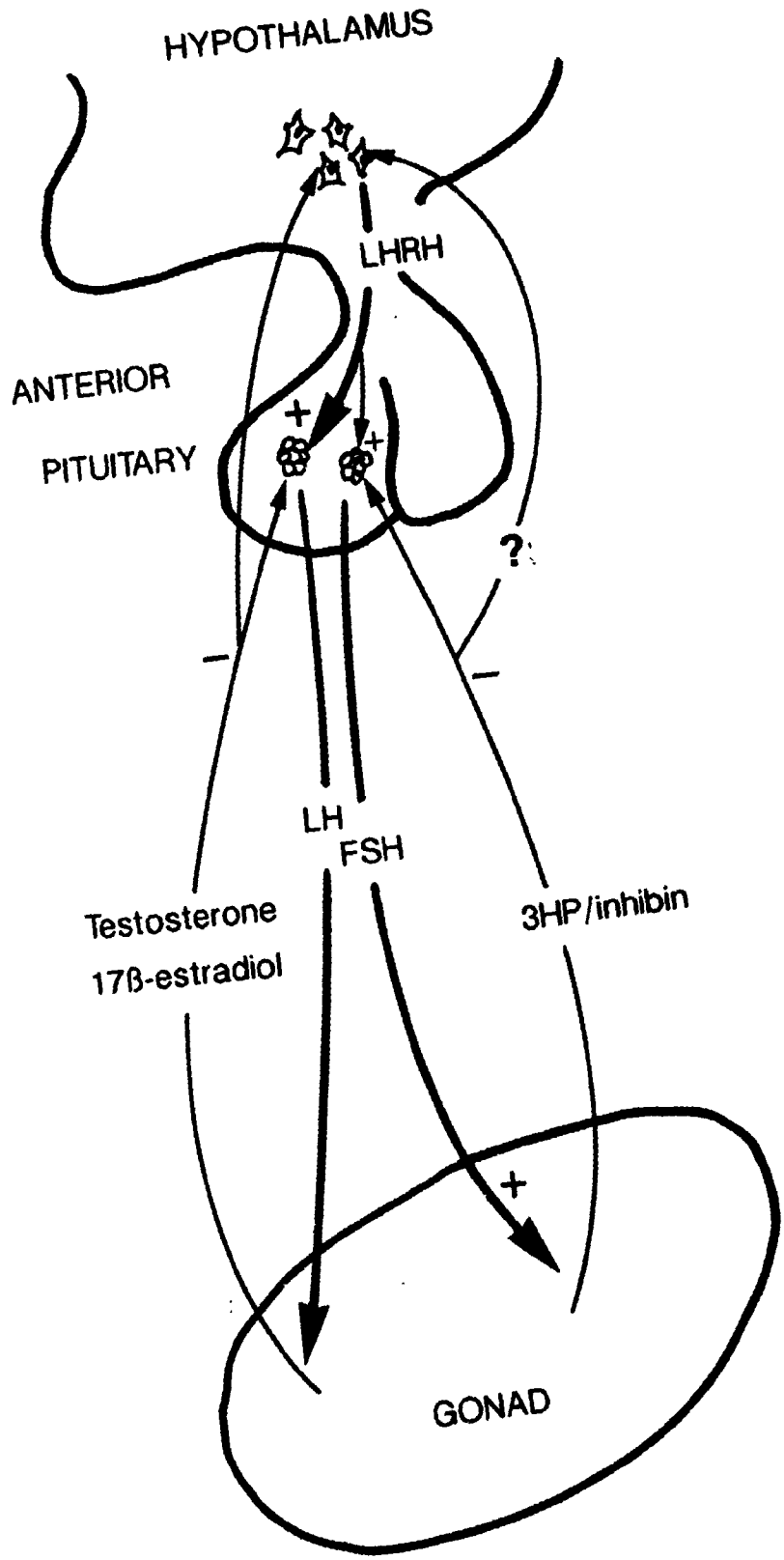
Until recently, evidence regarding the role of inhibin was largely circumstantial, relying on data such as Sertoli cell production, or rete testis fluid concentration, of inhibin as indicators of circulating inhibin since serum levels could not be detected (McLachlan et al, 1987). Other lines of evidence included the dependence of inhibin production on FSH and the ability of rather large amounts of follicular fluid or rete testis fluid to inhibit in vivo FSH secretion (Hermans et al, 1980; Thomas and Nikitovich-Winer, 1984). Measurement of inhibin has now been achieved and it appears that plasma inhibin is highest in the prepubertal male, 1800 fmole/ml, and relatively low in the mature adult, 400 fmole/ml (Rivier et al, 1988). deKretser and Robertson (1989) have shown that, in the adult, females have higher levels of circulating inhibin (10 U/ml) than males (1.5 U/ml). This recent work underscores the hypothesis that in the male, inhibin is important prepubertally to regulate FSH secretion while in the female, inhibin has a role in the

adult. Evidence regarding the role of 3HP in the regulation of FSH secretion is also somewhat circumstantial at this point in time. This line of thought relies on the production of 3HP by isolated Sertoli cells which is stimulated by FSH (Wiebe, 1982) and the effect of exogenous 3HP on FSH secretion both *in vivo* and *in vitro* (Wiebe and Wood, 1987; Wood and Wiebe, 1989). It seems likely then, that both 3HP and inhibin have roles in the regulation of FSH secretion and that perhaps each is primarily functional, or important, at different times or on different aspects of FSH secretion. Figure 34 presents a modified view of the negative feedback relationships in the hypothalamic-pituitary-gonadal axis of the rat. Work is progressing in this laboratory towards developing a specific antibody to 3HP and ultimately developing a specific radioimmunoassay to measure endogenous levels of 3HP, particularly with regard to establishing the likelihood that 3HP is capable of being transported to, or formed in, the brain and/or pituitary of the rat. 3HP has been detected in normal rat serum (Wiebe pers. comm.) and it is hoped that endogenous levels of 3HP will be correlated to circulating FSH and the age-related sensitvity to 3HP.

4.2.3 Specificity of 3HP Action

The FSH-suppressing activity of 3HP is related to the 3α-allylic and C_{20} keto configuration. Comparison of the *in vitro* activity of 3HP to structurally related steroids shows that none of the possible metabolites or variants of 3HP structure is capable of producing a similar selective FSH reduction *in vitro* (see Section 3.2.3). These results strongly suggest that 3HP itself is the active molecule and that it is not metabolized before acquiring activity as is the case with

Figure 34: A modified view of the hypothalamic-pituitary-gonadal axis in the rat. Luteinizing hormone-releasing hormone (LHRH) is secreted from the hypothalamus and is transported to the anterior pituitary via the hypophysial portal system. In the anterior pituitary, LHRH stimulates LH, and to a lesser extent FSH, secretion. LH stimulates steroidogenesis in the gonad and the products of this metabolism, testosterone or 17β-estradiol, provide negative feedback control of LH secretion at both the hypothalamic and pituitary levels. FSH stimulates gametogenesis and, more importantly here, the production of 3α-hydroxy-4-pregnen-20-one (3HP) and inhibin in the gonad. 3HP and inhibin are both capable of negatively-regulating pituitary FSH secretion, although 3HP suppresses FSH secretion more consistently than does inhibin. Whether either hormone regulates FSH secretion at the hypothalamic level is unknown at this time.


HYPOTHALAMUS
LHRH
ANTERIOR
PITUITARY
+
+
?
–
–
LH
FSH
Testosterone
17ß-estradiol
3HP/inhibin
+
GONAD

testosterone, which can be reduced to dihydrotestosterone or aromatized to 17β-estradiol (McEwen, 1981; MacLusky and Naftolin, 1981), or progesterone, which can also be metabolized before acquiring activity (Murphy and Mahesh, 1984a and b). Another line of corroborating evidence is that 3HP is effective *in vitro* at concentrations that may be considered physiological (10^{-16} and 10^{-14}M) (Dohler and Wuttke, 1975; Massicotte *et al*, 1984a and b).

Of some interest is the observation that all the structurally related steroids examined had no effect on FSH secretion *in vitro* and only affected LH secretion. Generally speaking, 3β-hydroxy-4-pregnen-20-one, 3α-hydroxy-5α-pregnan-20-one and 3β-hydroxy-5β-pregnan-20-one all increased the basal release of LH, while 3β-hydroxy-4-pregnen-20-one decreased, and 3α,20β-dihydroxy-4-pregnene increased the LHRH-induced release of LH, findings that have not been previously reported in *in vitro* studies. The C_{19} steroid structurally comparable to 3HP, 3α-hydroxy-4-androsten-17-one, only affected LH secretion (Table 7) suggesting that all 21 carbons are required for the FSH-suppressing activity of 3HP. These data further support the notion that 3HP is a physiological regulator of FSH secretion since there appears to be a specific receptor for 3HP. Confirming evidence may be obtained by identifying 3HP in rat serum.

The classic pharmacological approach, the evaluation of structure-function relationships, was employed in this study to examine the specificty of 3HP action. Current work in this laboratory is aimed at perfecting the synthesis of labelled (ie ^{3}H- or ^{14}C-) 3HP. Once a label is available a more direct approach may be taken, that is, specific binding of [^{3}H]-3HP to either whole pituitary homogenates or isolated

cells in culture can be assessed to determine the presence of specific 3HP receptors. Subsequently, any specific binding sites can be characterized. Metabolism of [^{14}C]-3HP in cultured pituitary cells can be examined to determine what the time-course and products of 3HP metabolism in the pituitary are.

4.3 DIFFERENTIAL REGULATION OF FSH AND LH SECRETION: A BRIEF COMMENT

Much attention has recently focussed on the differential regulation of the two pituitary gonadotropins, FSH and LH. Denef *et al* (1980) demonstrated that testosterone or dihydrotestosterone increased LHRH-induced FSH secretion in pituitary cultures and that this differential effect could be assigned to different functional subpopulations of gonadotropes. In the young male rat, pituitary content of LH increases as expected but FSH content decreases following castration (Vogel and Sherins, 1984). More information has been disclosed by treatment of castrate rats with LHRH antagonists. These studies show that LH secretion is decreased much more than FSH after LHRH antagonist treatment (ie. FSH is less dependent on LHRH than is true for LH) and that several steroids selectively affect FSH secretion only after treatment with LHRH antagonists (Bhasin *et al*, 1987; Kartun and Schwartz, 1987). Recent work with intact and castrate rats has shown that pulses of secreted LH closely follow those of LHRH while pulsatile FSH secretion is not directly related to LHRH pulses (Levine and Duffy, 1988). The finding in the present study that LHRH has a greater stimulating effect on LH than FSH also suggests that LHRH is not solely responsible for FSH secretion. McCann *et al* (1983) offer a good review of differential control of FSH secretion. The opinions on this topic are

not unanimous since Wise et al (1979) claim that some doses of LHRH selectively stimulate FSH secretion while Urbanski's group (1988) showed that FSH secretion does follow LHRH pulses suggesting that there is no differential regulation of pituitary gonadotropin secretion.

A number of suggestions as to how differential regulation occurs have been put forth: 1) a separate FSH-releasing factor may exist, but there is not much support for this hypothesis (Levine and Duffy, 1988; Urbanski et al, 1988); 2) considerable evidence supports the contention that basal FSH secretion is dependent on hypothalamic LHRH but that FSH pulsatility is independent of LHRH (DePaolo, 1985; Berardo and DePaolo, 1986; Culler and Negro-Vilar, 1987; Levine and Duffy, 1988); 3) different subcellular processing of the LHRH signal leading to separate effects on FSH and LH secretion (Levine and Duffy, 1988; Strobl and Levine, 1988; Kotsuji et al, 1988); 4) and related to number 3, gonadal steroids have differential feedback mechanisms where the intrapituitary response to LHRH is altered (Levine and Duffy, 1988; Strobl and Levine, 1988; Kotsuji et al, 1988). The work of Vogel and Sherins (1984) suggests that intracellular synthesis, processing and storage of gonadotropins may be the point of differential control. 3HP is a steroid capable of differential regulation of FSH secretion both in vivo and in vitro in a dose-dependent manner and may operate at the intrapituitary level in its heterogeneous effect on gonadotropin secretion.

4.4 INSIGHT INTO THE MECHANISM OF 3HP ACTION

In an effort to further examine the nature of 3HP action, activation and inhibition of the calcium and protein kinase C (PKC) signals of LHRH were carried out in the presence or absence of 3HP

and/or LHRH (Section 3.2.4).

A number of useful observations with respect to the calcium signal were obtained: 1) the calcium ionophore A23187 stimulates basal and LHRH-induced FSH secretion to a similar high level (a level greater than that achieved by LHRH alone) but does not increase LH secretion beyond the level attained with LHRH alone (Figure 29); 2) 3HP is inhibitory to the A23187-induced increase in secreted FSH; 3) inhibition of calcium uptake by verapamil or nifedipine (Figures 30 and 31) reduces the basal and LHRH-induced release of LH ; 4) FSH secretion is either not affected or actually increased by verapamil or nifedipine and, in the presence of both 3HP and verapamil or nifedipine, LHRH-induced FSH secretion drops sharply while basal FSH remains elevated under these conditions.

The first observation suggests that FSH secretion is less responsive than LH to the calcium signal produced by LHRH since the supranormal intracellular calcium concentrations produced by A23187 resulted in greater secretion of FSH, but not LH, than LHRH alone. 3HP opposes the effect of both LHRH and A23187 on FSH secretion suggesting that 3HP interacts with the calcium signal or subsequent cellular events. The third observation (along with the first) confirms the role of the calcium signal in the induction of LH secretion by LHRH (Conn et al,1987; Clayton, 1989). The fourth observation is the most surprising, that being that lowered intracellular calcium does not interfere with either basal FSH secretion or its induction by LHRH suggesting that FSH secretion is rather insensitive to lowered calcium levels and that LHRH may act on FSH via a primarily calcium-independent mechanism. 3HP is not effective in reducing basally secreted FSH in a low calcium environment but, together with verapamil or nifedipine, has a strongly negative

effect on LHRH-induced FSH secretion. The enhancement of the FSH-suppressing activity of 3HP by the calcium channel antagonists, verapamil and nifedipine, is somewhat similar to the enhancement or facilitation of the analgesic effect of 3HP when verapamil and nifedipine are administered parenterally and 3HP intracerebroventricularly (Wiebe and Kavaliers, 1988). All of this suggests that, unlike LH, basal FSH secretion is somewhat independent of calcium but can be stimulated by elevated intracellular calcium and further suggests that LHRH induction of FSH secretion does not occur chiefly via the calcium signal. As for 3HP, it appears that this steroid may be acting to increase the calcium requirement for FSH secretion, thus its apparent synergistic effect with the calcium channel antagonists and its opposing effect on LHRH- or A23187- induced FSH secretion. These insights further support the notion that there is, indeed, a differential control mechanism for the secretion of FSH and LH and that, similar to 17β-estradiol (Liu and Jackson, 1987), 3HP acts on the post-receptor mechanisms, particularly the calcium-generated post-receptor events, to selectively reduce LHRH-induced FSH secretion.

Protein kinase C (PKC) also plays a clear role in the secretion of gonadotropins <u>in vitro</u>. Attempted inhibition of PKC does not inhibit basal gondotropin secretion since, in the non-activated cell, basal PKC activity is already low (Nishizuka, 1986). Attempted PKC inhibition did, however, decrease the LHRH-induced secretion of both gonadotropins suggesting that PKC is not only involved in the long-term attenuation of the LH response to LHRH (Conn <u>et al</u>, 1987; Clayton, 1989) but plays a similar role for FSH. 3HP appears to interact with H-7 in suppressing FSH secretion (decrease in variability), although FSH levels are

suppressed to the level seen with 3HP alone and not further. Perhaps the interaction of 3HP with the calcium signal, with specific regard to FSH secretion, is also related to this interaction with PKC inhibition (H-7) since PKC activity is enhanced by calcium. PKC activation stimulates the basal release of LH as has been reported (Conn et al, 1987; Clayton, 1989). The decrease in LHRH-induced LH secretion brought about by PKC activation (with or without 3HP) may be due to an internal down-regulation of PKC (Nishizuka, 1986). PKC activation does appear to oppose 3HP inhibition of basal and LHRH-induced FSH secretion. These data suggest that 3HP does not act via an inhibition of PKC activity, but that 3HP can interact with PKC and that an active PKC component can antagonize the FSH-suppressing effect of 3HP.

The above studies into the mechanism of 3HP suppresssion of FSH have only scratched the surface and future studies will hopefully further illuminate the mechanisms governing differential secretion of FSH and the role of 3HP in this process.

Finally, 3HP is likely similar to other steroids in its action and plays a role in the ultimate regulation of FSH gene expression by one of the following mechanisms: suppression of FSH gene transcription; down-regulation of mRNA processing within the nucleus; inhibition of mRNA translation in the cytoplasm or increased degradation of FSH stored in secretory granules. Evidence on this point is indirect: 3HP selectively decreases FSH secretion in vivo and in vitro and decreases the cell content of FSH suggesting an overall reduction in FSH synthesis as well as release. Further investigations in this line (ie. the identification of a specific binding protein or receptor for 3HP localized to the nucleus of gonadotropes) should allow for more detailed studies such as

the incorporation of [3-H]-uracil into newly synthesized messenger ribonucleic acid for FSH before and after 3HP treatment, and the incorporation of [^{35}-S]-methionine into nascent FSH polypeptides under similar conditions.

SUMMARY

1) 3HP selectively suppresses FSH secretion *in vivo* in male and female, intact and castrate rats.

2) The FSH-suppressing effect of 3HP is dose-related and therefore is probably of physiological significance.

3) The senesitivity of the male rat pituitary to 3HP is in place by 10 days of age. 3HP has its greatest FSH-suppressing effect in the adult castrated male rat.

4) The *in vivo* FSH-suppressing effect of 3HP is unique compared to other gonadal and structurally-related steroids.

5) In anterior pituitary cells in culture, 3HP selectively inhibits both basal and LHRH-induced FSH secretion, without affecting LH secretion, at doses as low as 10^{-14}M.

6) The *in vitro* FSH-suppressing effect of 3HP is dose-related and occurs in the range of 10^{-16}-10^{-6}M 3HP, the physiological range for most steroids.

7) The action of 3HP *in vitro* is specific to its structure and is not due to the effect of a functionally or structurally-related steroid.

8) 3HP and inhibin probably act together, and perhaps at different

times, or on different aspects, to regulate FSH secretion in the rat.

9) The mechanism of the FSH-suppressing effect of 3HP most likely involves gene regulation (of the FSH gene) and appears to be concerned with the post-receptor signal of LHRH, specifically increasing the calcium requirement for, or otherwise altering the effect of increased intracellular calcium on, FSH secretion.

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