REGULATION OF ORNITHINE DECARBOXYLASE IN THE TESTIS OF RAT

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DOCTOR OF PHILOSOPHY

BY

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CERTIFICATE

This is to certify that Mrs. R. Madhubala has carried out the research work embodied in the present thesis under my supervision and guidance for the full period prescribed under the Ph.D. Ordinances of this University. I recommend her thesis entitled "REGULATION OF ORMITHINE DECARBOXYLASE IN THE TESTIS OF RAT" for submission for the degree of Doctor of Philosophy in this University.

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DECLARATION

I hereby declare that the work presented in this thesis has been carried out by me under the Supervision of Dr. P.R.K. Reddy and that this has not been submitted for a degree or diploma in any other University.

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Dated: July 16, 1982 **R. MADHUBALA**

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Dr. P.R.K.. REDDY
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To my husband

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ABBREVIATIONS

The following abbreviations are used in this thesis.

ABP	....	androgen binding protein
CAMP	....	dibutyryl 3'-5'-cyclic adenosine monophosphate
DFMO	....	a-difluoromethylornithlne
DNA	....	deoxyxibonucleic acid
DTT	....	dithiothreitol
EP	....	epinephrine
FSH	....	follicle stimulating hormone
HCG	....	human chorionic gonadotropin
INI	....	indomethacin
LH	....	luteinizing hormone
LHRH	....	luteinizing hormone-releasing hormone
MIX	....	1 methyl, 3-isobut yl-xanthine
NE	....	norepinephrine
ODC	....	ornithine decarboxylase (E.C. 4.1.1.17)
PB	....	phenoxybenzaraine
PG	....	prostaglandin
PMSG	....	pregnant mare serum gonadotropin
POPOP	....	(1,4-bis [2-(4-methyl-5-phenyloxazolyl benzene)]
PPO	....	2,5-diphenyloxazole
RNA,	....	ribonucleic acid
SD	....	standard deviation
SEM	....	standard error of the mean
TCA	....	trichloroacetic acid

C 0 N T E N T S

CHAPTER I

GENERAL INTRODUCTION AND REVIEW OF LITERATURE

GENERAL INTRODUCTION AND REVIEW OF LITERATURE

Several recent reports indicate that factors other than gonadotropic hormones may influence testicular function. Literature on the modulation of ovarian function by luteinizing hormone releasing hormone, catechoiamines and prostaglandins is available. However, comparatively little is known about the role of these hormones in male reproduction. In the present investigation the action of catecholamines, prostaglandlns, LHRH and gonadotroplc hormones on the testis of immature rat was studied using ornithine decarboxylase as a parameter. The literature on polyamines, prostaglandins, catecholamines, LHRH, and gonadotropic hormones is voluminous and is beyond the scope of this introduction for a comprehensive review. Hence only some relevant papers pertinent to the work reported in this thesis are reviewed as an introduction.

1.1 POLYAMINES:

The aliphatic diamine putrescine and polyamines spermidine and spermine are natural constituents of most living organisms. Though spermine was discovered by Leeuwenhoek in 1678, systematic research on these compounds was initiated only during the past two decades. Polyamines are distributed in all living organisms (Tabor and Tabor, 1964; Cohen, 1971).

However, the physiological significance and their role in various cellular processes are still not clearly understood. Recent literature shows that polyamines stabilize ribosomal structure (Cohen and Llchtensteln, I960; Norton et al,1968), bind to tRNA (Cohen, 1978), stimulate tRNA synthetase (Doctor et al, 1970), affect carbohydrate metabolism (Lockwood *et at*, 1971) and stabilize cell membranes (Liquori et al, 1967 3abbay *et* al, 1970 Silver et al, 1970). In addition to these effects a general regulatory role in transcription has also been suggested (Raina and Janne, 1970).

Williams-Ashman et al (1972) in their comprehensive review have elucidated the pathway leading to the formation of putrescine, spermidine and spermine in eukaryotic organisms, Putre seine is formed due to the dec arbo xylation of L-omithine by the enzyme L-ornithine decarboxylase. This appears to be the only pathway for the formation of putrescint in higher animal tissues. Putrescine in combination with the propyl amine moiety of the decarboxylated S-adenosyl methionine is converted into spermidine due to the enzymatic reaction catalyzed by spermidine synthetase. In a similar way spermi-dine and decarboxylated S-adenosyl methionine combine for the synthesis of spermine due to the action of spermine synthetase.

Ornithine decarboxylase is the rate limiting enzyme in the synthesis of polyamlnes. It is localized in the

soluble fraction of homogenates and requires pyridoxal phosphate. ODC appears to be not dependent on any metal ion for its activity. Rapid inactivation of ODC occurs in the absence of dithiothreitol due to polymerization of the enzyme (Janne and Williams-Ashman, 1971).

ODC has a pH optimum of about 7.0 and the Km for L-ornithine is roughly O.lmM.. It has an extremely short half life of less than 30 min (Russell and Snyder, 1969;| Janne and Williams-Ashman, 1970). It is inhibited by putrescine, spermidine, and spermine with an approximate KA of about lmM, 3mM and 9mM respectively (Pegg and Williams-Ashman, 1968; Janne and Williams-Ashman, 1971). These inhibition constants appear to be high for direct feed back inhibition. Beck and Canellakis (1972) proposed that the activity of ODC may be regulated by *an* intracellular protein, Canellakis and his co-workers have shown that a specific ODC-inhlbitory protein is induced in H-35 rat heptoma cells in vitro by the addition of putrescine to the culture medium (Fong et al, 1976) and this inhibitory protein was named as ODC-antizyme (Heller et al, 1976). However, it still needs to be established if this inhibitory protein is responsible for the regulation of ODC levels in vivo.

A number of hormones influence the levels of ODC in their respective target organs. Growth hormone increases and hypophysectomy decreases the levels of ODC in the liver

(Fausto and Butcher, 1976; Kostyo, 1966). In the thyroid gland thyroid stimulating hormone increases the levels of ODC (Richman et al, 1975; Scheinman et al, 1977). Epidermal growth factor produces an increase in ODC activity when added to epidermal cells in vitro (Stastny and Cohen, 1970) and nerve growth factor causes a marked increase in enzyme activity of brain in adult rats (Lewis et al,1978).

Pegg et al (1970) have demonstrated that the levels of ODC and S-adenosine methionine decarboxylase in the ventral prostate of rat are regulated by androgens. Similarly in the ovariectomised rat uterus and chick oviduct ODC levels were shown to be increased in response to estrogens (Cohen et al, 1970). The rise in ornithine decarboxylase of oviduct of chick in organ culture was abolished by simultaneous addition of cycloheximide (Cohen etal 1970).

In the rat ovary Kobayashi et al (1971) have observed that the levels of ODC are elevated 20 fold on the evening of proestrous. The above workers and Williams-Ashman et al (1972) showed that this rise in ODC activity in the ovary is induced by LH, hCG and PMSG. Subsequently it was demonstrated that LH causes de novo synthesis of ODC in the ovary, and that enhanced RNA synthesis may be required for this response (Kaye *et* al, 1973). In addition to the gonadotropic hormones prostaglandins were also shown to stimulate ODC activity in vivo in the ovary (Lamprecht et al, 1973) and in granulosa cells in vitro (Osterman and Hammond, 1978).

Mclndoe and Turklngton (1973) studied the levels of ODC and S-adenosyl methionine decarboxylase activity in the testis of rat at various stages of development. They observed very low levels of ODC on day 1 which rose rapidly reaching to maximal levels during days 10-17. This was followed by a decline in the activity of the enzyme and minimal levels of ODC were observed on day 28, The levels of ODC in the testis were shown to be regulated by FSH and LH and it was proposed that these hormones cause stimulation of this enzyme through CAMP (Reddy and Villee, 1975a).

The occurrence of polyamines in seminal plasma appears to be due to the secretory function of prostate and in several mammalian species prostate gland acts as a source for polyamines (Mann, 1964; Willlams-Ash man, 1965; Tabor and Tabor, 1964). Polyamines probably act as bacteriostatic agents and help in postejaculatory coagulation of seminal plasma in some of the species of mammals (Williams-Ashman et al, 1974).

1.2 INHIBITORS OF POLYAMINE SYNTHESIS:

The decarboxylases of ornithine and S-adenosyl methionine are the ideal targets for the inhibition of polyamine bio-synthesis. Methylglyoxal bis (guanylhydrazone), MGBG, is a potent and specific inhibitor of mammalian putrescine-activated

adenosylmethionine decarboxylase (Williams-Ashman and Schenone, 1972). a-Hydrazinoornithine (K_i of 2pM) and DL-o-hydrazino-6-amino valeric acid are the potent reversible inhibitors of ODC, The inhibitory effect of these compounds is abolished by high concentrations of pyridoxai phosphate. a-Methylornithine was found to be a potent competitive inhibitor (K_i of 20pM) of prostatic ODC (Abdel-Monem et al, 1974).

Among the compounds presently available, DL-a-dlfluoro-methyl ornithine (DFMO, RMI 71782) has proved to be the most potent inhibitor of ODC (Metcalf et al, 1978: Bey, 1978). DFMO works through the principle of substrate-induced irreversible inhibition (Rando, 1974; Bey, 1978). It does not inhibit other 1-carboxylases (Bey, 1978| Metcalf et al, 1978) and is non-toxic (the acute LD_{50} exceeds 3g/kg) and is devoid of acute pharmacological activity (Prakash et al, 1978; Fozard et al, 1980a). Administration of this compound to mice during eargly gestation suppressed the post implantation rise in uterine ODC and arrested embryonic development (Fozard et al, 1980b). It was also suggested that this drug may act as a postcoitally effective antifertility agent in female rats since it causes inhibition of embryogenesis at a very early stage (Reddy and Rukmini, 1981).

In male rats DFMO caused rapid, long lasting, dose dependent decrease of ODC activity in prostate and to a lesser extent in thymus and testis of rat. Repeated treatment of

DFMO decreased polyamine concentration in several rat tissues and selectively slowed down the growth of ventral prostate and thymus (Danzin et al, 1979a). Daily treatment of rats with DFMO from day 2 after birth until day 16 had no influence on net body growth or functional maturation. On the other hand adult rats given lg/kg of DFMO every 12h for 12 days showed a lesser gain of body weight than control animals (Seller et al, 1978), The work of Danzin et al (1979b) on ventral prostate showed that repeated injection of DFMO can totally block testosterone-indueed increase of putrescine, spermidine and RNA. However, no clear correlation was apparent between accumulation of polyamines and nucleic acids in these experiments.

1.3 ROLE OF PROSTAGLANDINS IN REPRODUCTION:

Prostaglandins constitute a class of naturally occurring 20 carbon unsaturated hydroxy fatty acids. Forty eight years ago an endogenous substance with vasodepressor and smooth muscle stimulatory activity was described in accessory genital glands and human semen by Goldblatt (1933)& von Euler (1934) who called it as prostaglandin. Several years later Bergstrom and Sjovall (1960a,b) isolated in pure from prostaglandin £ and prostaglandin F. In the following years, two independent groups demonstrated that prostaglandins are biosynthesized from polyunsaturated fatty acids (Bergstrom et al, 1964. VanDorp et al, 1964). The enzymes which synthesize prostaglandins are present in most organs; some organs such as

seminal vesicles, kidney and lung have a greater capacity for prostaglandin synthesis.

Vane (1971) and others discovered that the nonsteroid antiinflammatory drugs aspirin and indomethacin inhibit prostaglandin biosynthesis by the inhibition of the initial reaction in the conversion of arachidonic acid to prostaglandin. Thus the formation of the biologically active endoperoxides as well as prostaglandins is blocked by these drugs. In addition to aspirin and indomethacin the compounds which selectively antagonize the effects of prostaglandins at their site of action are 7-oxa prostaglandin analogues. Prostaglandin antagonists, however, do not inhibit all actions of prostaglandins and they are not highly potent.

Prostaglandins cause release of LH from anterior pituitary in vitro (Ratner et *al,* 1974) and in vivo (Carlson et al, 1973). Exogenous prostaglandins may act on the hypothalamus or higher centres to elicit LH release in the intact animal (Chatterjee, 1973). Prostaglandins of the E and F type were shown to be synthesized in the ovary of rat and rabbit (Zor et al, 1973; LeMaire et al, 1973). PGE mimics the action of LH in causing the induction of ovum maturation (Tsafriri et al, 1972a), stimulates adenyl cyclase (Marsh, 1971) and steroidogenesis (Speroff and Ramwell, 1970)* Stimulation of ovarian prostaglandin production by LH precedes follicular rupture and extrusion of mature oocyte (Tsafrlri

et al, 1972b) indicating the involvement of prostaglandins in this process. PGF_{2a} causes luteolysis of ovary and was suggested to be the uterine luteolytic factor responsible for regulating the functional life span of corpora lutea in most non-primate mammalian species (Goldberg and Ramwell, 1975). Prostaglandins cause contraction of human uterine smooth muscle (Bygdeman et al, 1968). which enables the use of these agents for termination of pregnancy.

Specific receptors for prostaglandins have been reported in the cell membranes of bovine corpus luteum (Rao, 1973).* Receptors for PGE appeared to be distinct from those of hCG (Rao, 1974). Recent findings show that PG binding sites are also present in intracellular organelles like rough endoplasmic reticulum, Golgi bodies and lysosomes of bovine corpora lutea (Rao and Mitra, 1977; Mitra and Rao, 1978a,b).

Though, prostaglandins were first isolated from the male, relatively little information is available on the role of prostaglandins in this sex. Mammalian testes are capable of prostaglandln synthesis and contain the enzymatic machinery necessary for its synthesis (Ellis *et al,* 1975)• Exposure of the testis to microgram quantities of prostaglandins can alter testicular blood flow (Free and Jaffe, 1972a), affect Leydig cells (Sebokova and Kolena, 1978), change the activity of enzymes regulating cholestrol metabolism (Takatori and Yamaoka, 1978), modify contractile activity of the seminiferous

tubules (Buhrley and Ellis, 1975) and the testicular capsule (Seeley et al, 1972) possibly resulting in the exfoliation of germ cells (Abbatiello et al, 1975; Tso and Lacy, 1975). Bartke et al (1973) showed that injection of PGF2α to intact mice caused decrease in testosterone levels in circulating plasma. Similarly prostaglandin implants significantly depressed serum testosterone, LH and FSH concentrations (Kimball et al, 1978). In addition to direct inhibition of steriodogenesis prostaglandinsalso reduce steroid synthesis in the testis by decreasing testicular blood flow (Free and Jaffe, 1972a; Free and Tillson, 1973; Einer-Jensen and Soofi, 1974). Prostaglandins inhibited LH, cAMP or MIX induced testosterone levels in dispersed rat testicular interstitial cells in vitro (Grotjan et al, 1978). However, Sairam (1979) showed that prostaglandins stimulate the levels of testosterone in the Leydig cells :in vitro.

1.4 CATECHOLAMINES:

Recent literature indicates an active role for catecholamines in ovarian and testicular function. The ovary exhibits adrenergic innervation (Owman et al, 1967). Systemic infusion of epinephrine stimulated progesterone secretion in women (Fylling, 1971) and adrenergic antagonists were shown to block ovulation in the rabbit (Virutamasen et al, 1977). Stimulation of steroidogenesis and cyclic AMP by epinephrine

in vitro was observed in the luteal tissue of rat (Harwood et al, 1979), cow (Condon and Black, 1976) and sheep (Jordan et al, 1978). Both rat and rabbit corpus luteura have been found to contain a gonadotropin-responsive adenyl cyclase system that is also responsive to β-adrenergic catecholamines (Birnbaumer *et al,* 1976). Since stimulatory effects of LH and catecholamines were not found to be additive it appears that the same adenyl cyclase system is responsive to both of these agents. Adenyl cyclase activity in the homogenates and in membrane particles from pig and rabbit follicles (Birnbaumeret al, 1976) was stimulated to a lesser extent by catecholamines indicating that the responsiveness to these agents develops with the formation of corpus luteum (Birnbaumer et al, 1976). Epinephrine induced adenyl cyclase activation and progesterone production was inhibited by propranolol, a β-adrenergic blocking agent (Harwood et al, 1980). However, phentolamine, the

α-adrenergic blocker had no effect. Catecholamines have also been shown to modulate the activity of ornithine decarboxylase in isolated porcine granulosa cells. This effect is most probably mediated by β-2-receptors linked to the adenyl cyclase system (Veldhuis et al, 1980).

Intravenous infusion of epinephrine reduced testosterone production rate in the humans (Levin *et* al, 1967) while intra-arterial infusion of either isoprenaline, epinephrine or

norepinephrine into the dog testis resulted in an increase in testosterone concentration in the venous effluent blood (Eik-Nes, 1969). These varied effects appear to be due to the effect of catecholamines on the blood vessels.

The possible role of catecholamines in Sertoli cell function has been examined in detail recently (Heindel et al, 1981). Catecholamines stimulate cAMP accumulation in the Sertoli cells and the β-antagonists inhibited the isoproterenol-induced CAMP accumulation. The α-adrenergic agonists or antagonists did not cause any change in the basal and isoproterenol-induced CAMP accumulation. Additional pharmacological studies revealed that theβ-receptor is of β_1-subtype in the Sertoli cells of rat (Heindel et al, 1981). Isoproterenol was also shown to stimulate the production of a protein kinase inhibitor (Tash et al, 1980) and accelarate testosteront metabolism by Sertoli cells (Verhoeven et al, 1979). The latter effect however required pharmacological doses of isoproterenol and was reported to be due to β_2-receptor stimulation (Verhoeven et al, 1979; Verhoeven, 1980).

15. LUTEINIZING HORMONE RELEASING HORMONE

Hypothalamic neurosecretory substances stimulate the release of luteinizing hormone and follicle stimulating hormone from the anterior pituitary gland. Secretory activity of LH and FSH were thought to be regulated by two discrete releasing

hormones namely luteinizing hormone releasing hormone (LHRH) and follicle stimulating hormone releasing hormone (FSHRH) respectively. However, following extensive purification, LHRH activity in the porcine (Schally et al, 1975) and ovine (Amoss *et al,* 1971) hypothalamic extracts could not be separated from FSHRH activity. Porcine LHRH was isolated by Schally et al, (1971), and its decapeptide chemical structure was proposed by Matsuo et al (1971a). Matsuo et al. (1971b) subsequently synthesized this hormone and it was shown that biologically it contained the properties similar to the natural LHRH (Arimura et al, 1972).

LHRH and many LHRH analogs stimulate the pituitary to cause synthesis and release of LH and FSH, thereby controlling all reproduction processes in both males and females. Thus processes such as onset of puberty, spermatogenesis follicular maturation, the periodicity of the estrous or menstrual cycle, ovulation and gestation are influenced by LHRH through the alteration in secretion rates of LH, FSH and gonadal steroids. In turn, gonadal steroids exert an inhibitory or stimulatory effect through negative or positive feedback mechanism on the release of LHRH, as well as on the pituitary LH and FSH responses to LHRH.

Though the principal action of LHRH appears to be on the pituitary, a number of recent studies have demonstrated that LHRH and its potent synthetic agonists act directly on

the reproductive organs (Sharpe, 1980). LHRH and its agonists cause inhibition of gonadotropic hormone induced response in the hypophysectomized rats (Rippel and Johnson, 1976; Hsueh and Erickson, 1979a,b; Ying and Guillerain, 1979). Hsueh and Erickson, (1979b) observed that LHRH and its agonists cause inhibition of FSH-induced increase in estrogen and progesterone secretion by rat ovarian granulosa cells in vitro. Ying and Guillemin (1979) demonstrated a dose dependent inhibitory effect by an LHRH agonist on gonadotropin-induced ovarian weight gain in hypophysectomized female rats.

In the male, LHRH and its analogs act directly on th® testis and cause a decrease in testicular LH/hCG receptors and inhibit testicular steroidogenesis in hypophysectomized male rats, indicating a direct effect on the Leydig cells (Hsueh and Erickson, 1979a). LHRH also causes inhibition of steroid induced increase in the weight of accessory reproductive organs of rat (Sundaram et al, 1981). The antiandrogenlc response of LHRH was also investigated on the mouse kidney (Lecomte et al, 1982). LHRH and its agonist inhibited testosterone induced increase in β-glucoronidase activity in castrated male mice.

All direct effects of LHRH on gonads may not be inhibitory. LHRH and its analogs increased PGE accumulation in ovarian granulosa cells (Clark et al, 1980). This stimulatory

effect of LHRH and its agonists appeared to be independent of the effects of LH or FSH. LHRH agonist appeared to stimulate prostaglandin accumulation in the granulosa cells by a mechanism independent of cAMP, Though earlier reports implicated CAMP as a mediator in the action of LHRH in the pituitary (Borgeat et al, 1972), recent studies have demonstrated that the release of LH and FSH in response to LHRH is independent of CAMP response (Naor et al, 1978; Conn et al, 1979; Sen and Menon, 1979).

Recent demonstration of specific, high affinity binding sites for LHRH and its agonists on luteal cells in the ovary (Clayton etal, 1979) and on Leydig cells in the testis (Reeves et al, 1980; Lefebvre et al, 1980) support the hypothesis that LHRH may have some regulatory role on the gonads. However, in the testis seminiferous tubules do not contain detectable number of specific binding sites for LHRH (Reeves et al, 1980). This indicates that the regulatory role of LHRH may be confined to only Leydig cells of the testis in males.

1.6 GONADOTROPIC HORMONES AND TESTICULAR FUNCTION:

During the postnatal development of the male growth and maturation of the testis is regulated to a large extent by the two glycopeptide hormones namely the luteinizlng

hormone and follicle-stimulating hormone. However, the exact role of these gonadotropins in the initiation and maintenance of spermatogenesis is still not clearly understood. FSH appears to play an important role during the initiation of spermatogenesis (Fritz, 1978) while LH facilitates the completion of spermatogenesis by stimulating the production of testosterone from the Leydig cells (Steinberger, 1971).

FSH initiates a series of biochemical events in the testis of immature rat (Means, 1974; 1975). FSH binds to specific receptors on the testis (Means and Vaitukaitis, 1972* Bhalla and Reichert, 1974| Steinberger and Chowdhury, 1974) and stimulates the adenyl cyclase system (Means, 1973). This leads to an increase in intracellular concentration of cyclic AMP (Murad et al, 1969; Kuehl et al, 1970a; Dorrington et al, 1972; Means, 1973; Dorrington and Fritz, 1974; Heindel et al, 1975), which in turn activates soluble protein kinase (Means, 1973; Means et al, 1974), The enhanced catalytic activity of this enzyme is responsible for the increase in phosphorylation of a variety of proteins (Means, 1975). The phosphorylated proteins appear to cause the subsequent biochemical effects. FSH has been demonstrated to stimulate general protein synthesis (Means and Hall, 1967, 1969, 1971; Abney et al, 1974; Dorrington et al, 1975) and also increase RNA synthesis (Means, 1971; Means and Tindall, 1975).

The main target cells for the action of FSH in the testis art Sertoli cells (Dorrington et al, 1974a, 1975). In addition to the actions reported above, FSH affects conversion of testosterone to 17β-estradiol, and stimulates DNA and androgen binding protein synthesis in Sertoli cells (Fritz et al, 1975). ABP is a 90,000 molecular weight substance and shows very high affinity for testosterone and 5α-dihydrotestosterone (Kd of approximately lnM) (Hansson et al, 1974; Vernon et al, 1974) ABP disappears following hypophysectomy and its replenishment requires injection of FSH (Hansson et al, 1973). In addition to FSH, androgens and LH cause enhancement of ABP in the testis (Hansson et al, 1976).

FSH is also implicated in the maturation of Leydig cells, and in the responsiveness of Leydig cells to LH (Odell at al, 1973* Chen et al, 1976; Van Beurden et .al, 1976). Administration of FSH to immature rats in vivo increases the number of LH receptors in the Leydig cells and increases the rate of testosterone production (Chen et al, 1976). These observations indicate that FSH acts primarily on immature testicular somatic cells and the influence of FSH on Leydig cells diminishes during gonadal maturation.

The primary target cells for LH in the testis are Leydig cells. Binding studies with ^{125}I-hCG/LH revealed that hormone binding is initially accompanied by increasing stimulation of testosterone synthesis. However, testosterone levels

reached a plateau when only a small proportion (<1%) of the total binding sites are occupied (Catt et al, 1974). Formation of CAMP is not detectable until testosterone production is almost maximal; synthesis and release of CAMP then proceeded in parallel with increase in hormone binding (Catt et al, 1974). This indicates that the Leydig cells contain a large number of excess receptors, above the number necessary for eliciting a maximum steroidogenic response.

Gonadotropic hormones are known to influence various enzymes in the testis. Teaticular intestitial tissue shows reduced Δ^{5}-3β-hydroxy steroid dehydrogenase (Levy *et al*, 1959) and (β-hydroxybutyrate dehydrogenase (Niemi and Ikonen, 1962). The activity of these enzymes is restored by injection of human chorionic gonadotropin (Samuels and Helmreich, 1956 Niemi and Ikonen, 1962). hCG also stimulates the conversion of progesterone to androgens in the hypophysectomlzed rat (Llaurado and Dominguez, 1963). Arvy (1962) has shown that hCG stimulates the proliferation of Leydig cells in the testis of immature rat and that hCG in the rat testis and LH in the chicken testis causes a marked increase in Δ^{5}-3β-hydroxy steriod dehydrogenase activity. Both hormones also stimulate a variety of other enzymes including acetyl esterase, β-glucornldase, sulfatase and 5-nucleotidase.

Hypophysectomy inhibits spermatogenesis and in the mouse testis it causes a fairly rapid and complete loss of

the testis specific LDH-X which disappears within about 3 weeks of the operation and parallels the loss of testis weight (Blackshaw and Elkington, 1967). Hypophysectomy of the rat affects esterase isozymes differently. The Organophosphate-sensltive activity of the majority of the interstitial tissue is sharply decreased after the operation but the c-type esterase of the Sertoli cells (acetyl esterase) and the organophosphate resistant but apparently p-chloromercuribenzoate-sensitive esterase of some of the intestitial cells appears to increase as these cells become chief surviors of the testis cell population (Niemi and Korraano, 1965; Niemi Niemi et al, 1966). Rukmini and Reddy (1981) have shown that glucosaraine-6-phosphate synthase is stimulated by FSH and PMSG while LH had no effect on this enzyme in the testis of immature rat.

1.7 DESENSITIZATIQNi

Prolonged exposure of the target organ to hormone may modify their response to subsequent stimulation by the same or by other hormones. Several instances have been described in which previous contact with physiological or high concentration of hormone results in a state of refractoriness or desensitization of the cells. This phenomenon has been widely documented for insulin (Roth et al, 1975), catecholamines (Remold-O'Donnell, 1974; Su et al, 1979; Anderson and Iaworskl, 1979), ACTH (Morera et al, 1978; Saez et al, 1979), parathyroid

hormone (Wong, 1979), vasopressin (Roy et al, 1976), prostaglandins (Zor et al, 1972 Lamprecht et al, 1973 Ciosek et al, 1975) and LH or hCG (Conti et al, 1977; Lamprecht et al, 1977; Tsuruhara et al, 1977; Saez et al, 1979). However, considerably less is known about FSH desensitlzation (Selstam et al, 1976; Weiss and Armstrong, 1979).

Refractoriness to hormones which act via adenyl cyclase system is associated with a marked decrease in hormone-stimulated cyclic AMP production, (Tell et al, 1978; Zor et al, 1976; Lamprecht et al, 1977; Selstam et al, 1976| Ahren et al, 1980). Prolonged stimulation of the target tissue with several hormones and catecholamines results in subsequent attenuation of CAMP response. The latter refractory phenomenon may be specific to the hormone used, so that agents capable of stimulating the accumulation of CAMP through other distinctive receptors are fully active (Plas and Nunez, 1975; Shear et al, 1976; Adachi et al, 1977; Lefkowitz and Williams, 1978); this is termed as receptor or agonist specific desensltization Studies on the agonist specific desensitization have mostly implicated alterations at hormone-receptor interaction and/or reduction in the number of receptors (Bockaert et al, 1976; Johnson et al, 1978; Harden et al, 1979; Saez et al, 1979; Conti *et al, 1971%* Lefkowitz and Williams, 1978; Wessels *et al,* 1978). On the other hand, stimulation by one hormone may cause the tissue not to respond to other normally effective agonists* this is generally termed as non-specific

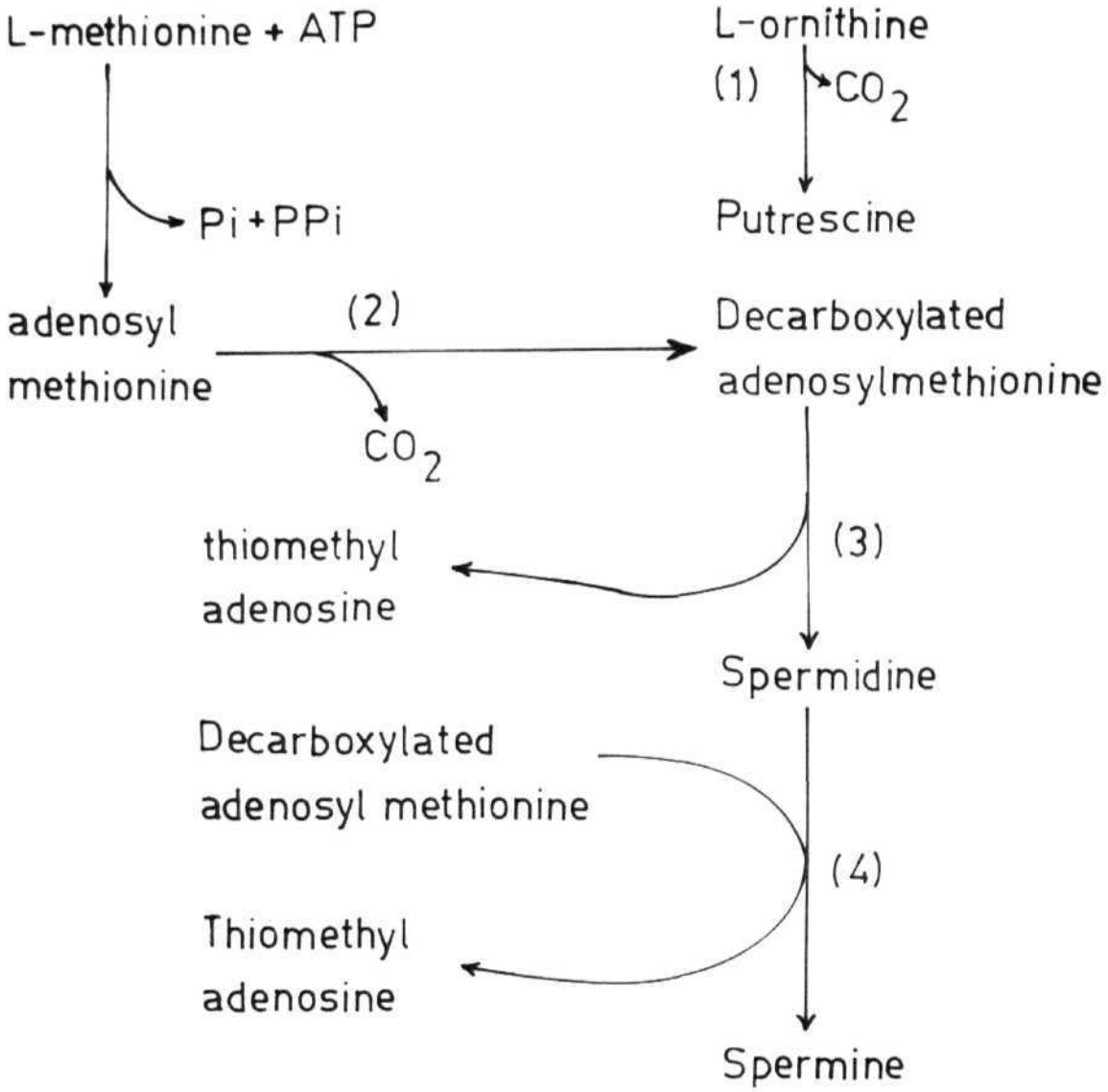
or heterologous desensitization (Newcombe et al, 1975; Su et al, 1916; Johnson et al, 1978). Heterologous desensitization most probably involves alterations at a step distal to the hormone receptor interaction (Johnson et al, 1978).

The extent of refractoriness depends both on the concentration of the agonist and the duration of the first stimulus. In response to the stimulus by an agonist the cells go through three phases of activity. Firstly an acute stimulation of adenyl cyclase, secondly on continued exposure, a gradual desensitization of cells and lastly a progressive recovery of responsiveness. However, this mechanism of desensitization is poorly understood.

1.8 SCOPE OF THE PRESENT INVESTIGATION:

This Thesis deals with a comprehensive account of the regulation of ornithine decarboxylase activity by prostaglandins, catecholamines, LHRH and gonadotropic hormones in the testis of immature rat. Using ODC as a parameter the phenomenon of desensitization of the testis to various hormones indicated above was also studied under various experimental conditions. In addition to these studies the effect of α-difluoromethylornithine, a specific inhibitor of ODC, on testicular function was studied in Immature and prepubertal rats.

Fig. 1 Scheme showing biosynthetic pathway of polyaraines in animal tissues. The enzymes catalyzing the reactions at various biosynthetic steps are given.



(1) Ornithine decarboxylase

(2) SAM-decarboxylase

(3) Spermidine synthase

(4) Spermine synthase

CHAPTER II

MATERIALS AND GENERAL METHODS.

MATERIALS AND GENERAL METHODS

2.1 CHEMICALS:

Ovine luteinizing hormone (NIH-LH-S-20), ovine follicle stimulating hormone (NIH-FSH-S-12) were a generous gift from National Pituitary Agency, National Institute of Arthritis, Metabolism and Digestive Diseases (NIAMDD), Bethesda, U.S.A. Highly purified FSH (100XNIH-FSH-SI) was generously provided by Dr. M.R. Sairam, Clinical Research Institute of Montreal, Canada. Prostaglandins were provided by the Upjohn Company, Kalamazoo, Michigan, U.S.A. Dibutyryl 3',5'-cyclic adenosine monophosphate (cAMP), indomethacin (IM), ornithine, pyridoxal phosphate, dithiothreitol (DTT), tris base, reduced glutathione, collagenase (Type IV), bovine serum albumin, 2,5-diphenyloxazole (PPO), 1,4-bis [2-(4-methyl-5-phenyloxazolyl)] benzene (POPOP), luteinizing hormone releasing hormone, L-epinephrine bitartrate, L-norepinephrine bitartrate, tyramine hydrochloride, serotonin hydrochloride, isoproterenol bitartrate, DL-propranolol hydrocholoride, dopamine hydrochloride, aspirin, orcinol, calf thymus deoxyribonucleic acid (DNA) and yeast ribonucleic acid (RNA) were purchased from Sigma Chemical Company, St. Louis, MO, U.S.A. [^{3}H]L-Leucine (3,300 mCi/mmol) and [^{3}H]-thymidine (9,800 mCi/mmol) were obtained from Bhabha Atomic Research Centre, Trombay, India. DL-[1-^{14}C] Ornithine monochloride (58mCi/mmol) was purchased from the Radiochemical Centre, Amersham. Hyamine hydroxide (tissue solubilizer) was obtained

from Beckman. 1 Methyl, 3-isobutyl-xanthine (MIX) was obtained from Aldrich Chemical Company. Cyclic AMP assay kit was purchased from the Radiochemical Centre, Amersham. Phenoxybenzamine (PB) and practolol were obtained from Smith Kline and French (India) Limited, α-Difluoromethylornithine (DFMO) was generously provided by Dr. David J, Wilkins of Centre de Recherche Merrell International, Strasbourg, France. All other chemicals were of analytical grade and were purchased locally.

2.2 ANIMALS:

Immature male rats, derived from Wistar strain, aged 21-22 days, weighing between 25-30g were used in this study. The rats were given water and the pellet diet from Hindustan Lever Ltd., India, ad libitum. They were housed in an air conditioned room (25°) and a regimen of 14h light and 10h dark cycle was maintained.

2.3 HORMONE TREATMENT:

Animals were injected intratesticularly using a Unimatrix syringe with a 27 gauge needle as described by Reddy and Villee (1975b). Prostaglandins, gonadotropins, luteinizing hormone releasing hormone, cAMP, 1 methyl, 3-isobutylxanthine, indomethacin were injected in a total volume of 5-10µl per testis in saline, ethanol or in

saline:ethanol (9:1, v/v) mixture. α-Difluoromethylornithine was injected in saline intraperitoneally. Aspirin was injected intraperitoneally in propylene glycol. [^{3}H] Thymidine and [^{3}H] L-leucine were injected in saline intratesticularly. Catecholamines and other neurotransmitters were also administered in saline intratesticularly. In all experiments the control animals received an equal amount of vehicle. Most of the inhibitor studies were done by injecting the inhibitor 15 min before the treatment of the hormone.

2.4 PREPARATION OF ENZYME SAMPLES:

The rats were killed by spinal dislocation. Testes were removed, decapsulated and homogenized in 4 volumes of ice cold TED buffer (Tris, 25mM; EDTA, 0.1mM; DTT, 1.0mM; pH 7.4) in an all glass homogenizer (Kontes) and centrifuged at 25,000 x g for 30 min in a MSE refrigerated centrifuge. The supernatant was used for the assay of ornithine decarboxylase activity.

2.5 ASSAY OF ORNITHINE DECARBOXYLASE ACTIVITY:

The assay for ODC activity was done essentially as described by Janne and Williams-Ashman (1971). The assay mixture contained 0.5μmoles of unlabelled ornithine, 2.5μmoles of dithiothreitol, 0.1μmoles of pyridoxal phosphate and 0.2μCi of radioactive ornithine (250,000 cpm) and 200μl of enzyme extract in a final volume of 0.5ml. For studies

involving isolated Leydig cell and seminiferous tubule fractions 0.25μ moles of unlabelled ornithine was used. The incubations were carried out in glass tubes equipped with rubber stoppers from which polyethylene center wells (Kontes Glass Co.) or glass wells containing 0.2ml of hyamine hydroxide were suspended. The tubes were incubated at 37°C for 60 min in a metabolic shaker and the reaction was stopped by injecting 0.5ml of 10% TCA. The tubes were reincubated for an additional 30 min to trap all liberated $^{14}CO_2$. The center wells were removed and placed in scintillation vials containing 10ml of scintillation mixture (4.0g PPO; 0.2g POPOP in 1 litre of toluene). The samples were counted using Beckman Liquid Scintillation Spectrometer (Model L5 3133P). The enzyme activity is expressed as p moles of CO_2 liberated per mg protein per hour,

2.6 ASSAY OF CYCLIC AMP a

Preparation of tissue extract: The rats were killed by cervical dislocation and testes were removed and frozen in liquid nitrogen. The frozen testes were transferred to a glass homogenizer with a teflon pestle (Potter-Elvejhem type) at -20°. Ten volumes of ice cold 10% Trichloroacetic acid were added and the mixture was immediately homogenized. After it had stood for 10 minutes at 0° it was centrifuged for 20 minutes at 3000 x g at 0-3°. The supernatant solution was transferred to a glass centrifuge tube and extracted six times with 2 volumes of water saturated diethylether. Following the last ether extraction the solution was heated

in a water bath at 100°C for i-2 minutes until the odour of ether disappeared. cAMP in the neutralized extract was stable indefinitely at -20°.

Cyclic AMP Assay Procedure: The Radiochemical Centre cyclic AMP assay kit is specific and sensitive. The assay was performed in small tubes suitable for centrifugation. All assay tubes were kept at 0°C in an ice-water bath. To the assay tubes containing the standard or the unknown, 50µl of labelled [^{3}H] cAMP (0.025µCi) and 100µl of the binding protein were added. Known amount of cAMP (2pmol-16pmol) and varying aliquots of unknown samples were added to the assay tubes. A total volume of 50µl is typically reserved for these components. To determine blank counts 150µl of 0.05M tris-EDTA buffer, pH 7.5, containing 4mM EDTA was taken in duplicate tubes containing labelled cAMP. All the tubes were vortex mixed for about 5 seconds and the ice bath containing the tubes was kept in a cold room at 2-4°C and left for 2 hours. At the end of this period 100µl of charcoal suspension was added, mixed briefly and the tubes were replaced in ice bath. The tubes were centrifuged for about 5 min to sediment the charcoal. 200µl of sample from each tube was removed and placed in scintillation vials containing Bray's mixture (4g of PPO, 0.2g POPOP, 60g napthalene, 20ml ethyleneglycol, 100ml of methanol made upto 1 litre with dioxane). The vials were counted in a Beckman Liquid Scintillation Spectrometer

(Model LS 3133P). The ratio of the c.p.m. bound in the absence of unlabelled cAMP (Co) to the c.p.m. bound in the presence of standard or unknown unlabelled cyclic AMP (Cx) was calculated. From the Co/Cx value for an unknown sample, the number of pmoles of cAMP were calculated using the standard curve. The cAMP activity is expressed as pmoles/ml of extract.

2.7 ISOLATION OF LEYDIG CELLS AND SEMINIFEROUS TUBULES :

The decapsulated testes from 6-8 animals were pooled and Leydig cells and seminiferous tubules were separated by the method of Moyle and Ramachandran (1973)• The testes were incubated in 50ml stoppered centrifuge tubes containing Krebs-Ringer-bicarbonate buffer (0.5ml/testis), pH 7.4, 0.1% collagenase and bovine serum albumin and lOmM glucose. Addition of lmM reduced glutathione to the buffer increased the stability of ODC; hence it was added to the buffer during incubation. The incubation was carried out for 30 min by placing the tubes in a water bath maintained at 37°C. The tubes were shaken with a frequency of 75 cycles/min for 30 minutes and 0.15M NaCl was added to double the volume of Krebs-Ringer bicarbonate buffer. The tubes were inverted gently several times and allowed to stand for 15 min at room temperature. The turbid supernatant solution contained the Leydig cells while the seminiferous tubules remained at the

bottom. The supernatant containing Leydig cells was then filtered through nylon gauze and centrifuged at 100xg at room temperature for 10 min to sediment the Leydig cell fraction. Leydig cells and seminiferous tubules were homogenized in 4 vol of TED buffer (25mM tris, 0.1mM EDTA and 1mM DTT), pH 7.4, in an all glass homogenizer and centrifuged at 25,000xg for 30 min. The supernatant was used for the assay of ODC activity,

Krebs-Ringer solution:

100 parts of 0.9% NaCl (0.15M)
4 parts of 1.15% KCl (0.15M)
3 parts of 1.22* $CaCl_2$ (0.11M)
1 part of 2.11% KH_2PO_4 (0.15M)
1 part of 3.8% $MgSO_4$ $7H_2O$ (0.15M)

Krebs-Ringer bicarbonate buffert

16 ml of 1.3% $NaHCO_3$ made upto 100 ml with Krebs-Ringer solution and gassed with CO_2 and pH adjusted to 7.4

2.8 EXTRACTION AND ESTIMATION OF NUCLEIC ACIDS:

The nucleic acids were extracted according to the procedure of Schmidt and Thannhauser (1945) as per the modification of Munro (1966). Two millilitres of 10*

homogenate (w/v) of the testes was mixed with 2.5ml of 10% ice cold TCA and centrifuged to remove acid soluble compounds. The sediment was washed once with 2.5ml of 10/. ice-cold TCA. After the removal of the acid soluble compounds the sediment was extracted twice with 5ml of 95% ethanol and the extract was removed by centrifugation. An alcohol-ether (3:1) wash was given to the sediment to remove any traces of lipids. The lipid free tissue was suspended in 2ml of 1N KOH and incubated for 2 hours at 37°C. DNA and protein were then precipitated by addition of 0.4ml of 6N HCl and 2.6ml of 5% TCA and allowed to stand in ice for 10 minutes and centrifuged. The supernatant fraction was collected separately to estimate RNA content. The sediment was suspended in 2.5ml of 5% TCA and boiled at 90°C for fifteen minutes with occasional shaking. The mixture was centrifuged and the supernatant collected in a test tube. The sediment was washed with 1.5ml of 5% TCA and the combined supernatants were taken for the estimation of DNA.

Estimation of Nucleic Acids:

DNA was estimated by the diphenylamine colour reaction and RNA by the orcinol reaction (Schneider, 1957). For estimation of DNA one ml of the DNA extract was mixed with 2ml of diphenylamine reagent and heated for 10 minutes in boiling water. The intensity of blue colour was read at 600nm in a Systronics Spectrophotometer. The amount of DNA

present in a sample was determined from a standard curve using calf thymus DNA as the standard.

For the estimation of RNA one ml of RNA extract was diluted to 1.5ml with 5% TCA and heated for 30 min after adding 1.5ml of orcinol reagent in a boiling water bath. The intensity of the green colour was then read at 700nm. A standard curve was prepared using purified yeast RNA as the standard.

Diphenylamine reagent: This was prepared by dissolving one gram of purified diphenylamine in 100ml of glacial acetic acid and 2.75ml of conc. H_2SO_4.

Orcinol reagent: One gram of purified orcinol was dissolved immediately before use in 100ml of concentrated HCl containing 0.5g of $FeCl_3$.

2.9 ESTIMATION OF PROTEIN:

Protein content was estimated by the method of Lowry et al (1951). Different aliquots of the unknown sample were taken and made up to 1ml with distilled water. To this 5ml of alkaline copper reagent was added (1ml of 2% sodium potassium tartrate and 1ml of 1% copper Sulfate mixed with 100ml of 2% sodium carbonate in 0.1N sodium hydroxide) and the contents were mixed. The tubes were allowed to stand for 10 minutes and 0.5ml of 1N Folin-Ciocalteu reagent was added.

The colour was measured after 30 min at 670nm against the blank. The protein concentration of the sample was determined from a standard curve using 20-200µg of crystalline bovine serum albumin (fraction IV) as standard.

2.10 INCORPORATION OF [^{3}H]-LEUCINE AND [^{3}H]-THYMIDINE INTO TCA PRECIPITABLE MATERIAL:

Incorporation of [^{3}H]-leucine or [^{3}H]-thymidine into TCA precipitable material was determined at 4 hours after the injection. 5µCi of the precursor was given intratesticularly under mild ether anesthesia. At autopsy, testes were removed and homogenized in 2.0ml of distilled water and precipitated by the addition of an equal volume of cold 10% TCA. The precipitate was centrifuged at 2000xg for 10 min. The resulting pellet was washed twice with cold 5% TCA followed by one wash with 1:1 mixture of ethanol and ether. The pellet thus obtained was dissolved in 0.2ml of hyamine hydroxide and counted in Beckman Liquid Scintillation Spectrometer (Model LS 3133P) containing 10ml of Bray's solution.

2.11 STATISTICAL ANALYSIS:

Student's 't' test was used for analysis of the data obtained in different experimental groups.

CHAPTER III

A. REGULATION OF ORNITHINE DECARBOXYLASE ACTIVITY BY PROSTAGLANDINS IN THE TESTIS OF IMMATURE RAT.

B. DESENSITIZATION OF TESTICULAR ORNITHINE DECARBOXYLASE TO PROSTAGLANDINS

REGULATION Of ORNITHINE DECARBOXYLASE ACTIVITY BY PROSTAGLANDINS IN THE TESTIS OF IMMATURE RAT

INTRODUCTION

Prostaglandins appear to cause several effects in the male reproductive system. They mimic the action of LH and stimulate the synthesis of testosterone (Sairam, 1979) and cAMP (Grotjan et al, 1978) in dispersed rat interstitial cells. However, in mice (Bartke et al, 1973) and rats (Saksena et al, 1974) injection of prostaglandins was also shown to cause decrease in testicular androgen production.

In females, prostaglandins induced ovum maturation (Tsafriri *et* al, 1972a) and stimulated adenyl cyclase levels (Marsh, 1971) and steroidogenesis (Speroff and Ramwell, 1970), It was also observed that the in vivo levels of ODC in the ovary of rat (Lamprecht et al, 1973) and in granulosa cells in vitro (Osterman and Hammond, 1978) were increased following exposure to prostaglandins. These compounds have been found to play an intermediary role in the action of gonadotropic hormones in the ovary (Kuehl et al, 1970b). However, the intermediary role of prostaglandins in LH or FSH action in the male is not known. Reddy and Villee (1975a) observed that gonadotropic hormones and cAMP stimulate the levels of ODC in the testis of immature rat. The present work shows that PGE_2 and $PGF_{2\alpha}$ mimic yet another action of gonadotropic

hormones and cause stimulation of ODC activity in the testis of immature rat.

MATERIALS AND METHODS:

The source of the chemicals and animals is given in Chapter II. Stock solutions of prostaglandins were prepared in ethanol at a concentration of 10mg/ml and diluted with 9 volumes of saline before use. Stock and working solutions of hormones, cAMP and 1 methyl, 3-isobutyl xanthine (MIX) were made in saline. The animals were injected intratesticularly using a Unimatrix syringe with a 27 gauge needle. Prostaglandins, FSH, LH, cAMP or MIX were injected in a total volume of 5-10µl per testis. Control animals received 10µl of saline, 5µl of ethanol or both as per the experimental design. At an appropriate time the animals were killed by spinal dislocation and testes were dissected out. The decapsulated testes were homogenized in 4 volumes of cold homogenizing buffer in an all glass homogenizer (Kontes) and centrifuged at 25,000xg for 30 minutes. The supernatant was used for the assay of ODC activity. The methods for determination of protein content and ODC activity are described in detail in Chapter II.

For isolated cell studies the decapsulated testes from 6-8 animals were pooled and Leydig cells and seminiferous tubule fractions were separated by incubation in Krebs-Ringer bicarbonate buffer containing collagenase as described

in Chapter II. ODC activity of the separated fractions was measured as described in the proceeding chapter.

In experiments involving the effect of PGE_2 and $PGF_{2\alpha}$ on cAMP content animals were injected intratesticularly with PG or MIX or both. After 30 minutes the animals were killed and the testes were removed and frozen in liquid nitrogen* The frozen testes were processed as in Chapter II and the supernatant was used for the assay of cAMP. cAMP was assayed by using cyclic AMP assay kit purchased from Amersham (Chapter II). The cAMP content is expressed as pmoles/ml of extract.

RESULTS

The levels of ODC in the testis at various ages from day 10 to day 75 are given in Fig. 1. The enzyme activity on day 10 of age was found to be high. At later time intervals ODC activity declined gradually reaching to minimal detectable levels at day 75. Day 21-22 animals were used for all subsequent experiments as the testes at this age were highly responsive to various hormonal manipulations.

The effect of PGE_2 at a dose of 10µg/testis on the activity of OX at various time intervals is given in Fig. 2. The enzyme activity increased significantly at 1h reaching to maximal levels at 2h after the injection. This was followed

by significant reduction at 4h declining to control levels at 6h.

The effect of different doses of PGE_2 on ODC activity is shown in Fig. 3. An increase in the activity of the enzyme was observed following treatment with 5µg of PGE_2, reaching maximal levels at a dose of 10µg per testis. Higher doses of PGE_2 caused less stimulation in the activity of the enzyme.

The effect of PGE_2 and $PGF_{2\alpha}$ alone and PGE_2 in combination with cAMP or MIX is given in table 1. Injection of MIX to prostaglandin treated animals caused additional stimulation of ODC activity when compared with the animals injected with MIX or PGE_2 alone. cAMP alone caused an increase in the activity of ODC. Injection of PGE_2 to cAMP treated animals induced additional stimulation of the enzyme. $PGF_{2\alpha}$ did not cause enhanced stimulation of ODC in the PGE_2 injected animals

The effect of PGE_2 and $PGF_{2\alpha}$ alone and PGE_2 in combination with MIX on cAMP levels is shown in Fig. 4. PGE_2 and $PGF_{2\alpha}$ caused a significant increase in cAMP at 30 minutes after the injection. The cyclic nucleotide phosphodiestrase inhibitor, MIX at a dose of 5µg/testis also caused significant increase in cAMP levels. In addition MIX potentiated PGE_2 stimulated increase of cAMP,

The effects of LH, FSH individually or in combination with PGE_2 is given in table 2, The gonadotropic hormones caused significant stimulation of ODC activity at a dose of 20µg per testis. Injection of PGE_2 at a dose of 5 or 10µg per testis to the LH or FSH treated animals caused additional stimulation of ODC activity.

The effects of PGE_2 and $PGF_{2\alpha}$ alone and PGE_2 in combination with FSH or LH on ODC activity in the isolated Leydig cells and seminiferous tubules of testis is given in table 3. It was observed that PGE_2 and $PGF_{2\alpha}$ significantly stimulated the activity of ODC in both Leydig cells and seminiferous tubules. LH caused significant stimulation of the enzyme activity in Leydig cells while FSH increased the ODC levels in seminiferous tubules only. Treatment with FSH and PGE_2 did not increase the levels of ODC in the seminiferous tubules over the levels seen in animals treated with PGE_2 alone. Similarly injection of LH and PGE_2 did not cause hyperstimulation in the Leydig cell fraction. This could be due to the use of saturating dose of PGE_2 which by itself would have caused stimulation of ODC activity in these two fractions.

DISCUSSION

The present study shows that during the development of rat testis the levels of ODC are altered. Elevated levels of

polyamines are associated with embryonic, cancerous and other actively growing tissues (Russell, 1973). High levels of ODC observed in early developmental stages could be due to the active growth and development of the testis. McIndoe and Turkington (1973) demonstrated high ODC activity on day one after birth when the seminiferous tubules contain only gonocytes and Sertoli cells. They also observed a decrease in ODC activity during the maturation stages of testicular development. The decrease in ODC activity observed in this study during the development and differentiation of the testis could be due to the rapid proliferation of the cells which do not contain ODC; resulting in low specific activity of the enzyme.

It is interesting to observe that PGE_2 and $PGF_{2\alpha}$ like FSHt LH or cAMP cause stimulation of ODC activity in rat testis. These results are similar to the observation made earlier on the effect of PGE_2 on rat ovary *in vivo* (Osterman and Hammond, 1978) and *in vitro* (Lamprecht *et al*, 1973). Studies on the time courses of prostaglandin stimulation of ODC revealed that maximum stimulation occurs at 2h after the treatment. This effect is dose dependent.

The mechanism of action of prostaglandins in stimulation of ODC in the testis is not well understood. Prostaglandins were shown to cause an increase in the levels of cAMP in

the testis (Grotjan et al, 1978; Kolena, 1975). This effect is similar to th* action of gonadotropic hormones on the tt$tis (Dorrington et al, 1972; Heindel et al, 1977). Reddy and Villee (1975a) and Osterman et al (1978) postulated that the gonadotropic hormone induced increase in the levels of ODC in the testis and granulosa cells is probably mediated through cAMP. In this study it was observed that PGE_2 and $PGF_{2\alpha}$ cause stimulation of cAMP levels at 30 min after the injection of these drugs while ODC levels were increased at a much later time of 2h. Furthermore, the group treated with cAMP in combination with PGE_2 showed significantly high ODC activity when compared to cAMP alone group and in addition in the group treated with MIX and PGE_2 the enzyme levels were significantly high compared to PGE_2 or MIX alone group. These observations further support the hypothesis that the elevated concentrations of cAMP in the testis increase the levels of ODC, supporting the hypothesis that the action of prostaglandins is probably mediated by cAMP. However, it is possible that other additional mechanisms for the stimulation of ODC by prostaglandins are also operative in the testis.

It was Interesting to note that treatment with FSH or LH along with maximally effective dose of PGE_2 caused additional stimulation of ODC activity. Hyperstimulation of ODC activity observed in the present study following treatment with saturating levels of PGE_2 and FSH or PGE_2 and LH may be due to the combined action of these compounds. It is also possible that

PGE_2, LH and FSH are acting on different cell types and are causing additional ODC activity. Studies using isolated cells shows that hypers timul ation of OX levels following treatment with PGE_2 and gonadotropic hormones observed in whole testis appears to be due to the combined effect of these compounds on different cell types in the testis of rat. $PGF_{2\alpha}$ in combination with PGE_2 did not cause any additive effect. This is probably due to the action of these compounds on similar types of cells in the testis. These results show that in addition to gonadotropic hormones, prostaglandins also appear to play an important role in the regulation of some of the metabolic functions of the testis.

TABLE I

EFFECT OF PGE_2, $PGF_{2\alpha}$, cAMP AND MIX ON ORNITHINE DECARBOXYLASE ACTIVITY IN TESTIS

Prostaglandins, MIX and cAMP were injected directly into the testis in 5-10µl of vehicle as described in general materials and methods section and the animals were killed after 2h. Results are mean ± S.E.M. of 3-5 determinations from 6-10 animals per group,

Groups	Treatment	ODC activity (pmoles/h/mg protein)
1.	Control	455 ± 9
2.	PGE_2 (10µg)	1315 ± 123
3.	$PGF_{2\alpha}$ (10µg)	1152 ± 24
4.	PGE_2 (10µg) + $PGF_{2\alpha}$ (10µg)	950 ± 125
5.	cAMP (20µg)	726 ± 55
6.	PGE_2 (10µg) + CAMP (20µg)	997 ± 51[a]
7.	MIX (5µg)	804 ± 61
8.	MIX (5µg) + PGE_2 (10µg)	1902 ± 129[b]

a-significantly different from group 5 ($p<0.01$)

b-is significantly different from group 7 ($p<0.01$) and group 2 ($p<0.05$)

TABLE II

EFFECT OF COMBINED TREATMENT WITH PGE_2 AND GONADOTROPIC HORMONES ON ODC ACTIVITY IN THE TESTIS

Hormones and PGE_2 were administered intratesticularly in 5-10µl of vehicle as described in materials and methods section. Animals were killed 2h after the injection. Results are mean ± S.E.M. of 3-5 determinations from 6-10 animals per group.

Groups	Treatment	ODC activity (pmoles/h/mg protein)
1.	Control	455 ± 9
2.	PGE_2 (10µg)	1315 ± 125
3.	PGE_2 (5µg)	969 ± 15
4.	LH (20µg)+PGE_2 (5µg)	1527 ± 123[a]
5.	LH (20µg)	1354 ± 34
6.	LH (20µg) + PGE_2 (10µg)	2082 ± 93[b]
7.	FSH (20µg)	793 ± 42
8.	FSH (20µg) + PGE_2 (5µg)	1125 ± 71[c]

a-significantly different from group 3 ($p<0.001$)
b-significantly different from group 5 ($p<0.001$)
c-significantly different from group 7 ($p<0.01$)

TABLE III

EFFECT OF PGE_2, $PGF_{2\alpha}$, FSH AND LH ON OX ACTIVITY IN IN THE ISOLATED LEYDIG CELLS AND SEMINIFEROUS TUBULES OF TESTIS

All animals were killed at 2h after the injection of prostaglandins (10µg) or at 4h after the injection of LH (40µg) or FSH (40µg). The animals in groups 4 and 6 were treated with PGE_2 for 2h after the administration of LH or FSH. The values are mean ± S.E.M. of 4-5 different observations consisting of 8-10 animals in each group.

Group No.	Treatment	ODC (pmoles/h/mg protein) Leydig cells	Seminiferous tubules
1.	Saline	322 ± 18	549 ± 40
2.	PGE_2	970 ± 133[d]	1852 ± 228[d]
3.	LH	540 ± 75[a]	643 ± 69
4.	LH + PGE_2	859 ± 79[d]	1116 ± 176[b]
5.	FSH	360 ± 46	1006 ± 176[a]
6.	FSH + PGE_2	753 ± 292[a]	1033 ± 144[c]
7.	$PGF_{2\alpha}$	1012 ± 110[d]	1277 ± 338[c]

[a] $p<0.05$; [b] $p<0.02$; [c] $p<0.01$; [d] $p<0.001$ as compared to group 1.

Fig, 1 Pattern of omithine decarboxylase in the testis during various ages of rat. The values represent mean ± S.E.M. of 4–5 determinations from 8-10 rats per group.

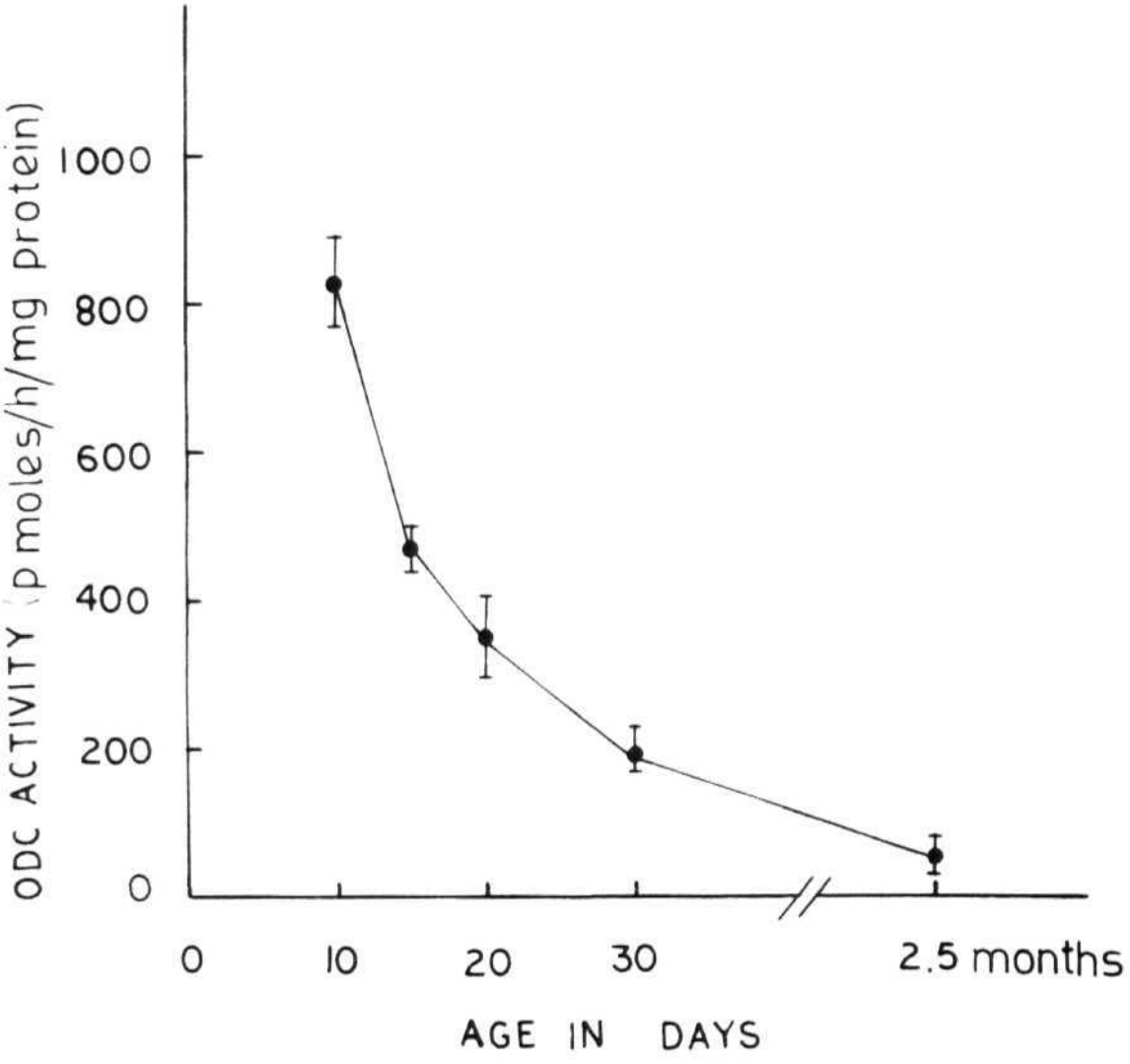
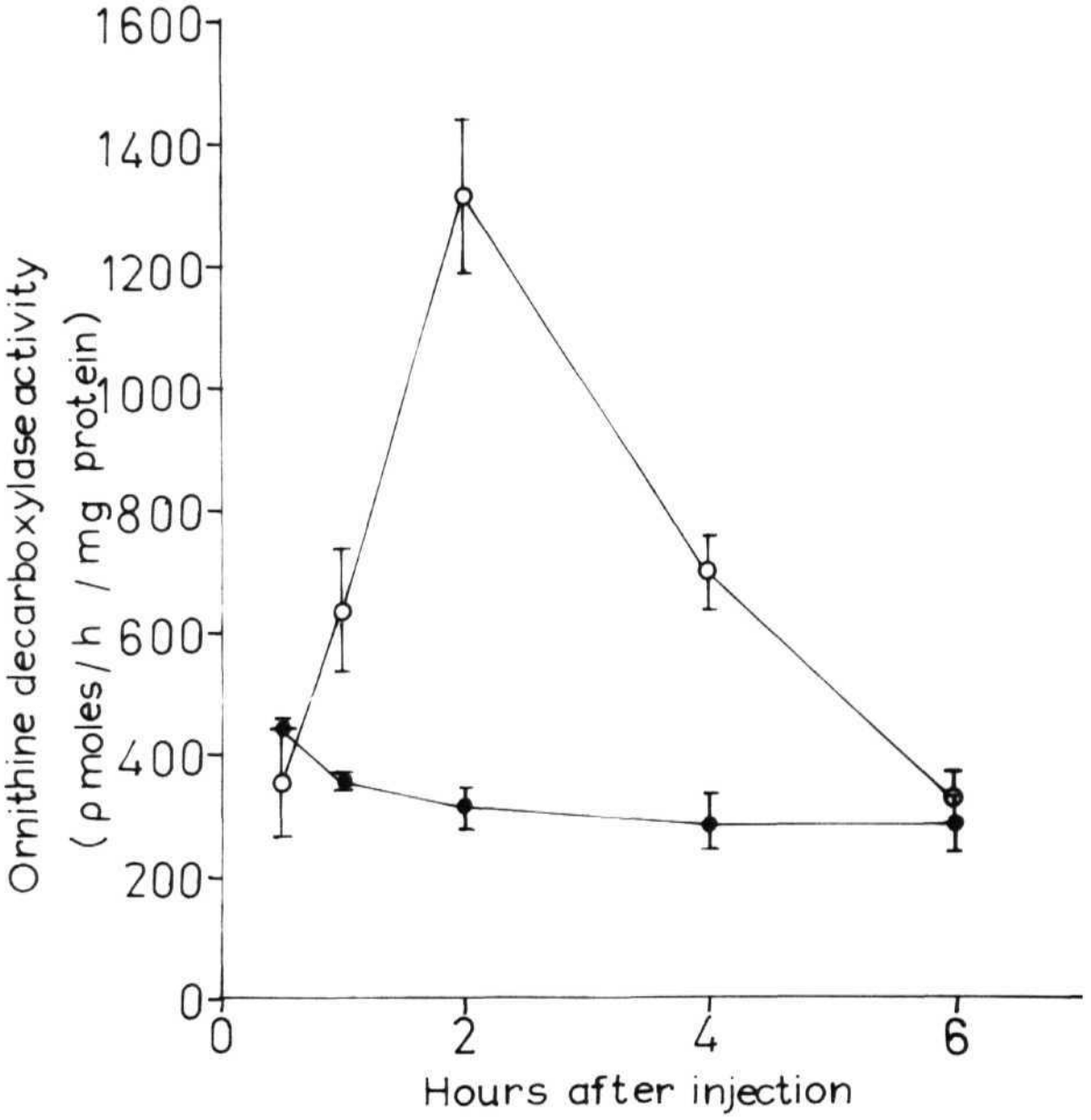

ODC ACTIVITY (p moles/h/mg protein)
1000
800
600
400
200
0
0
10
20
30
2.5 months
AGE IN DAYS

Fig. 2 Effect of PGE_2 on the levels of ODC activity in the testis of rat at various time intervals. PGE_2 was injected intratesticularly at a dose of 10μg per testis. Each point represents mean ± S.E.M. of 3–5 determinations from 6–10 animals. O——O PGE_2, ●——● saline.


Ornithine decarboxylase activity
(p moles / h / mg protein)
1600
1400
1200
1000
800
600
400
200
0
0
2
4
6
Hours after injection

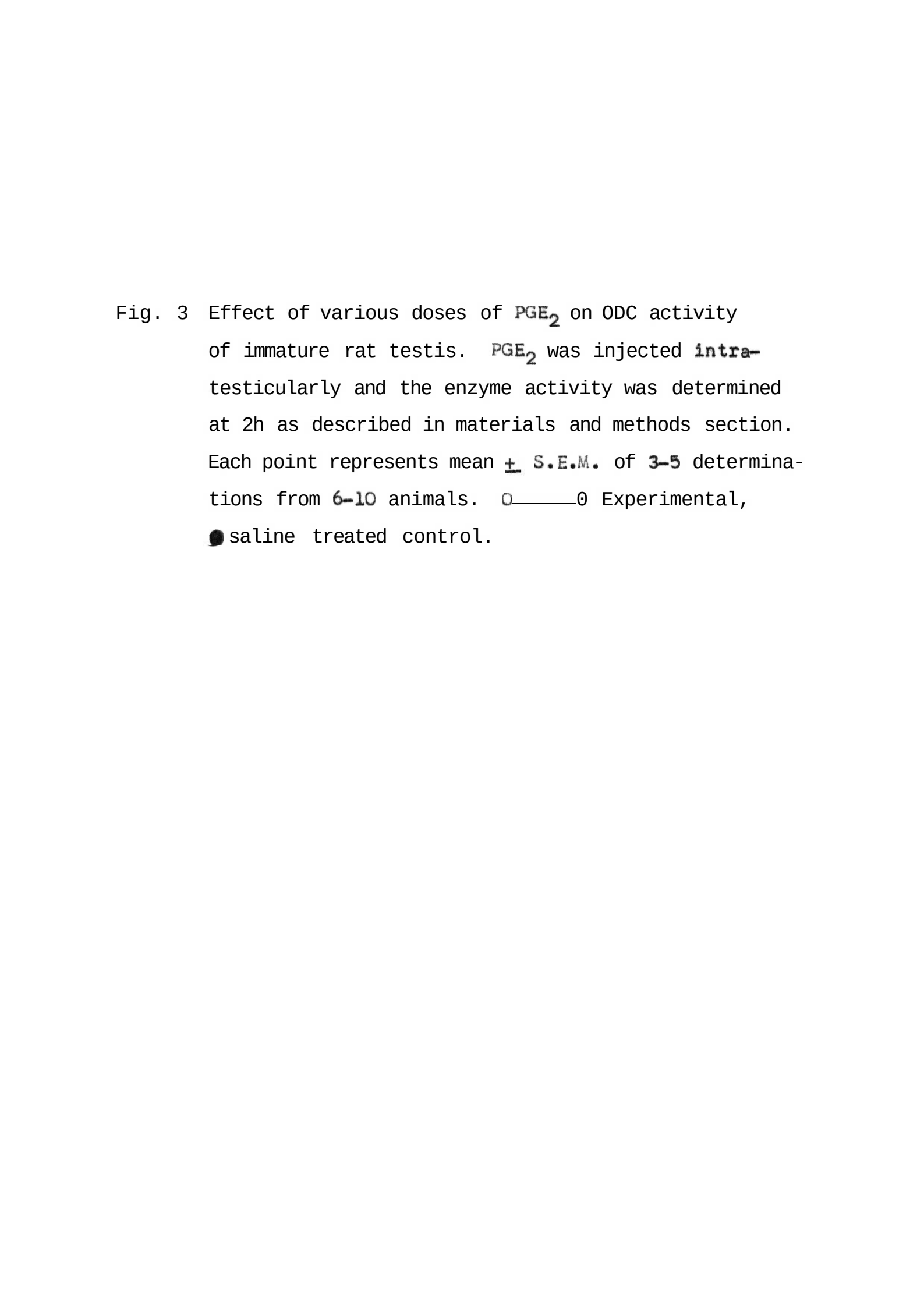


Fig. 3 Effect of various doses of PGE_2 on ODC activity of immature rat testis. PGE_2 was injected intratesticularly and the enzyme activity was determined at 2h as described in materials and methods section. Each point represents mean ± S.E.M. of 3–5 determinations from 6–10 animals. O——O Experimental, ● saline treated control.

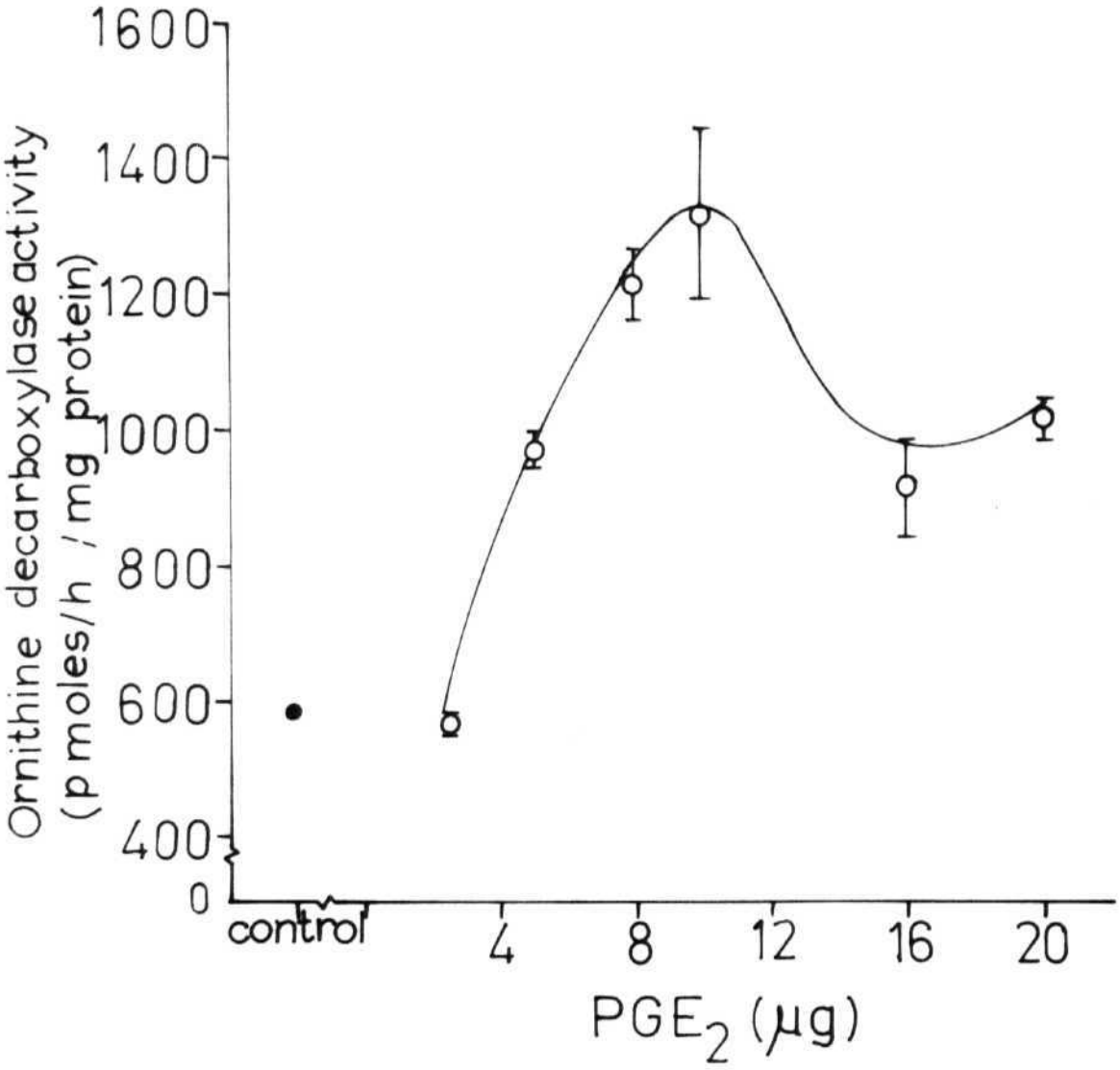
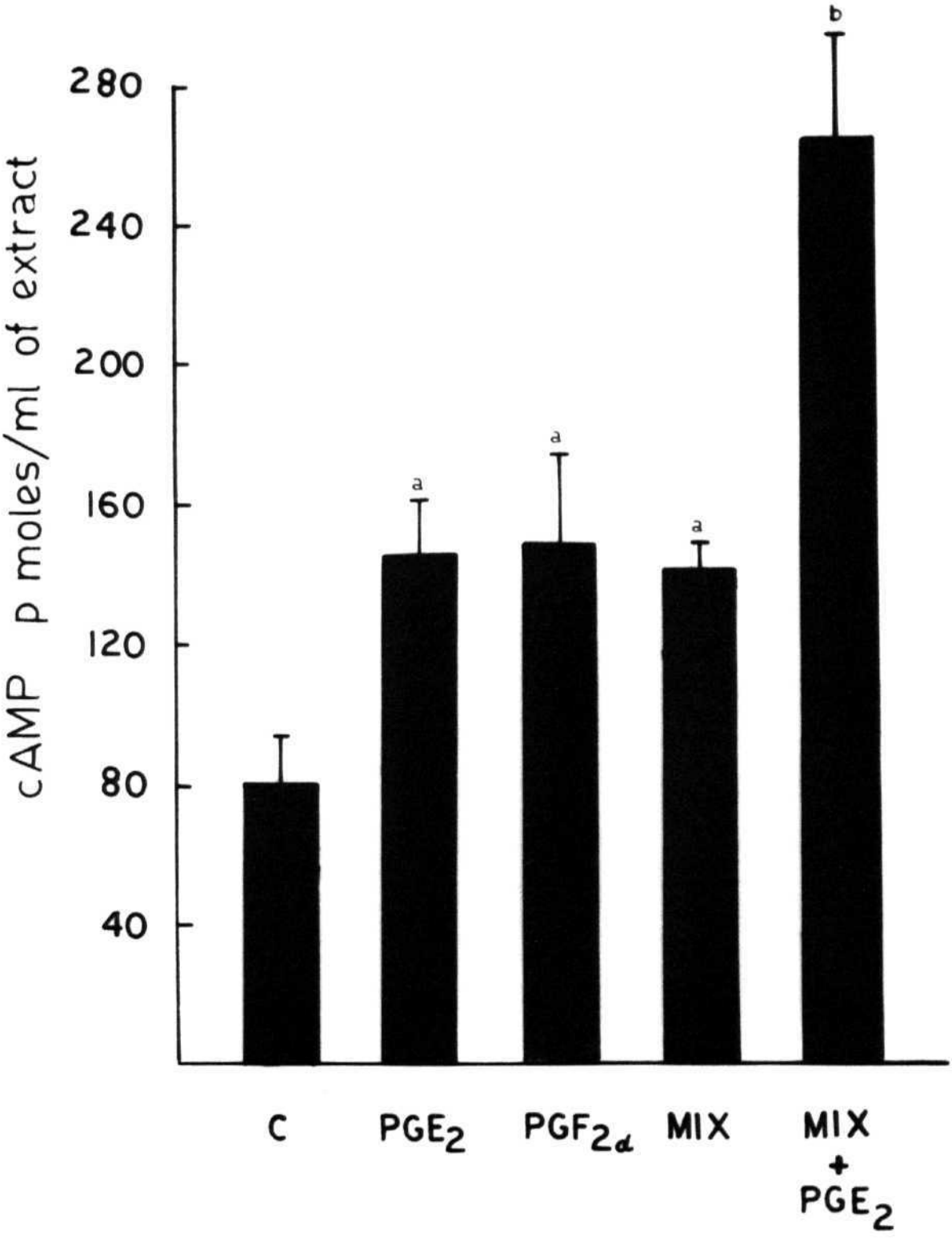

Ornithine decarboxylase activity
(p moles/h / mg protein)
1600
1400
1200
1000
800
600
400
0
control
4
8
12
16
20
PGE2 (µg)

Fig. 4 Effect of PGE_2, $PGF_{2\alpha}$ alone and PGE_2 in combination with MIX on the levels of cAMP in immature rat testis. PGE_2 and $PGF_{2\alpha}$ were injected intratesticularly at a dose of 10μg per testis. MIX was injected at a dose of 5μg per testis, cAMP content was determined at 30 rain after the injection of above compounds as described in materials and methods section. Each point represents mean ± S.E.M. of 4 values. C-represents saline injected control. a-Significantly different from control ($p<0.001$—0.01), b-Significantly different from PGE_2 alone treated group ($p<0.001$).


cAMP p moles/ml of extract
280
240
200
160
120
80
40
a
a
a
b
C
PGE2
PGF2α
MIX
MIX + PGE2

DESENSITIZATION OF TESTICULAR ORNITHINE DECARBOXYLASE TO PROSTAGLANDINS

INTRODUCTION:

Several tissues have been shown to decrease in hormonal responsiveness following continued exposure to homologous hormone (Catt et al, 1979a; Tell et al, 1978). Such a phenomenon known as desensitization is wide spread in biological tissues.

Prolonged exposure of prostaglandins to intact cells renders these cells refractory to renewed exposure to prostaglandins in terras of cAMP formation (Lamprecht et al, 1979). However, the precise mechanism of desensitization to prostaglandins is not clear. In Chapter III A it was shown that prostaglandins cause stimulation of ODC activity in the testis of immature rat and that this stimulation of ODC activity by prostaglandins is mediated by cAMP. Refractoriness to hormones which act via adenyl cyclase system is associated with a marked decrease in hormone stimulated cAMP production (Zor *et* al, 1976| Lamprecht et al, 1979). In this study ODC was used as a marker to investigate the phenomenon of PGE_2 desensitization in the testis of immature rat.

MATERIALS AND METHODS:

The source of chemicals is given in Chapter II. Immature

male rats were given injections intratesticularly in a total volume of 5-10µl. At the desired time rats were killed by spinal dislocation and the decapsulated testes were homogenized in 4 volumes of homogenizing buffer (Chapter II) and centrifuged at 25,000 x g for 30 min and the supernatant was used for the assay of ODC activity. The methods for the determination of ODC activity and protein content are described in Chapter II. In some experiments Leydig cells and seminiferous tubules were separated by collagenase treatment and the QDC activity of the isolated cells was estimated as described in Chapter II. For the assay of cAMP content the animals were killed by spinal dislocation and the testes were removed and frozen in liquid nitrogen. The frozen testes were processed for extraction and estimation of cAMP as in Chapter II.

RESULTS;

The effect of time on desensitization of ornithine decarboxylase response in the testis is given in Fig. 1. In these experiments PGE_2 at two doses of 10 and 25µg was used to study the phenomenon of desensitization in the testis. Following the injection of the animals intratesticularly with PGE_2 at 0 time a second injection of the stimulatory dose of 10µg was given similarly at 4, 16, 24 and 48h and the animals killed at 2h after the second

injection to monitor ODC activity. The results show that exposure of the testis to 10 or 25µg of PGE_2, 4 and 16h previously, did not render the testis refractory to second injection of PGE_2 and indicating that the testis was not desensitized at these time intervals. However, following injection with 10 and 25µg of PGE_2 the testis was desensitized at 24h as the second injection with 10µg of PGE_2 did not cause stimulation of ODC activity. The testis became partially responsive at 48h in the group desensitized with 25µg of PGE_2 only. In these experiments appropriate controls were treated with vehicle alone at 0 time followed by a second injection with PGE_2 at 4, 16, 24 and 48h. In these animals the ODC levels were elevated and were similar to the group treated with PGE_2 alone indicating that the desensitization effects observed in Fig. 1 are not due to the saline-ethanol injection used as vehicle or due to the stress of first injection. Experiments to observe restimulation with PGE_2 at later time intervals were not conducted as ODC of the testis was found to become less responsive to PGE_2 and gonadotropic hormones after day 23.

The effect of various doses of PGE_2 on desensitization of testicular ODC is shown in Fig. 2. In these experiments various doses of PGE_2 were injected intratesticularly and at 24h thereafter the stimulatory dose of 10µg of PGE_2 was injected to monitor ODC levels after a further interval of 2h.

The results show that a minimum of 10µg of PGE_2 is necessary to cause desensitization of testis in terms of ODC activity. At higher doses of 25 and 50µg the testis showed much more diminished response to the second injection of PGE_2 indicating that the effect of desensitization is also dose dependent. In addition to PGE_2, $PGF_{2\alpha}$ also caused desensitization of testis. The results presented in Fig. 3 show that a minimum of 25µg of $PGF_{2\alpha}$ per testis is necessary to cause desensitization of the testicular ODC response.

Fig. 4 shows the effect of LH, FSH, and CAMP following desensitization with 10µg of PGE_2. At 24h after intratesticular injection of PGE_2, 40µg of FSH or LH or 20µg of cAMP were injected similarly and the testicular ODC in these groups was measured subsequently at 2h. The results show that in PGE_2 desensitized testis both the gonadotropic hormones and cAMP do not stimulate OX activity as the ODC values were significantly low when compared to FSH, LH and cAMP alone treated controls respectively.

The effect of PGE_2 desensitization on Leydig cells and seminiferous tubules is given in Fig. 5. In these experiments at 24h after the injection with 10µg of PGE_2 a second injection of PGE_2, FSH, LH or cAMP was given. The animals were killed 2h thereafter and the testicular seminiferous tubules and Leydig cells were separated by collagenase treatment.•

ODC in the two fractions was estimated separately and the data compared with respective controls. The results show that in desensitized testis second injection with PGE_2 does not cause stimulation of ODC in both Leydig cells and seminiferous tubules. Similarly cAMP also did not stimulate enzyme activity following PGE_2 desensitization in both the testicular fractions. The ODC activity of PGE_2 desensitized seminiferous tubule fraction was significantly less following FSH injection when compared to animals treated with FSH alone. Similarly the ODC activity of Leydig cells in the PGE_2 desensitized testis was significantly less following a second injection with LH when compared to the controls treated with LH alone.

Fig. 6 shows the effect of various doses of $PGF_{2\alpha}$ on desensitization of testis using cAMP as the parameter. At 24h after the injection of varying doses of $PGF_{2\alpha}$ a second injection of 10µg of $PGF_{2\alpha}$ was given intratesticularly and the levels of cAMP were estimated at 30 min in the total testis. A dose of 50µg of $PGF_{2\alpha}$ was found to be necessary to cause desensitization of cAMP response in the testis.

DISCUSSION;

The results presented in this study show that prolonged exposure of the testis to PGE_2 or $PGF_{2\alpha}$ renders the testis refractory to second treatment with PGE_2 or $PGF_{2\alpha}$. Refractoriness to PGE_2 was dose and time dependent. The testes which

were refractory to PGE_2 progressively regained their responsiveness to PGE_2 at a later time in terms of ODC activity.

It was also observed that prolonged exposure of the testis to PGE_2 or $PGF_{2\alpha}$ not only rendered the testis refractory to these homologous agents but also caused desensitization to LH, FSH, and cAMP, the other known ODC stimulating agents in the testis. This type of desensitization is known as 'cross desensitization' or heterologous desensitization and was observed earlier for various other hormones (NewCombe et al, 1975; Su et al, 1976; Johnson et al, 1978; Tougui et al, 1980). However, in rat granulosa cells LH caused stimulation of cAMP following PGE_2 desensitization (Lamprecht et al, 1979).

In the preceding section it was suggested that the action of PGE_2 and $PGF_{2\alpha}$ in stimulating ODC activity is probably mediated through cAMP. However, in this section it was observed that while $PGF_{2\alpha}$ causes desensitization of ODC activity at a dose of 25µg, this compound causes desensitization of cAMP response at a much higher dose of 50µg per testis. If the ODC activity is totally mediated through cAMP the lower dose of 25µg of $PGF_{2\alpha}$ must have caused desensitization of both cAMP response as well as ODC response. Hence it appears that the stimulation of ODC in response to prostaglandins is complex and could be due to some other additional metabolic changes.

The mechanism of desensitization caused by prostaglandins is not clear. Incubation of frog erythrocyte membrane preparation with PGE_2 caused a fall in receptor number (Lefkowitz *et al*, 1977). It has also been suggested that a protein inhibitor triggered by PGE_2 may be responsible for the development of refractoriness in the ovarian follicle (Lamprect *et al*, 1979). In addition to this it was observed that the cytoskeleton of the cell plays an important role in the adenylate cyclase response to PGE_2 and stabilization of tubulin prevents development of refractoriness to PGE_2 (Zor *et al*, 1979). Desensitization of testis following injection with PGE_2 observed in this study may be due to any one or all of the above factors.

It was interesting to observe that cAMP injection to PGE_2 desensitized testis did not cause stimulation of ODC activity in the testis. This indicates that the lesion caused by the first injection of PGE_2 is beyond cAMP production. A post-cyclic AMP block to testicular steroidogenesis following hCG desensitization was observed and this block was correlated with a decrease in the steroidogenic enzyme activity in the microsomes of Leydig cells (Chasalow *et alt* 1979). This study shows that ODC synthesis is disturbed at a step beyond cAMP formation following desensitization with PGE_2.

Fig. 1 Effect of time on desensitization of ornithine decarboxylase response to PGE_2 in the testis. Following injection with 10 or 25µg of PGE_2 at 0 time a second injection of 10µg of PGE_2 was given at 4, 16, 24 or 48h. Controls (0- - - -0) were treated with vehicle alone at 0 time followed by a second injection with PGE_2 at 4, 16, 24 or 48h. Arrow represents ODC activity of animals treated with vehicle alone at 0 time and at 24h later. All animals were killed at 2h after the second injection of vehicle or PGE_2 respectively for the assay of ODC activity. Values represent mean ± S.E.M. of 3-5 determinations from 6–10 animals in each group.

Fig. 1 Effect of time on desensitization of ornithine decarboxylase response to PGE_2 in the testis. Following injection with 10 or 25µg of PGE_2 at 0 time a second injection of 10µg of PGE_2 was given at 4, 16, 24 or 48h. Controls (0- - - -0) were treated with vehicle alone at 0 time followed by a second injection with PGE_2 at 4, 16, 24 or 48h. Arrow represents ODC activity of animals treated with vehicle alone at 0 time and at 24h later. All animals were killed at 2h after the second injection of vehicle or PGE_2 respectively for the assay of ODC activity. Values represent mean ± S.E.M. of 3-5 determinations from 6-10 animals in each group.

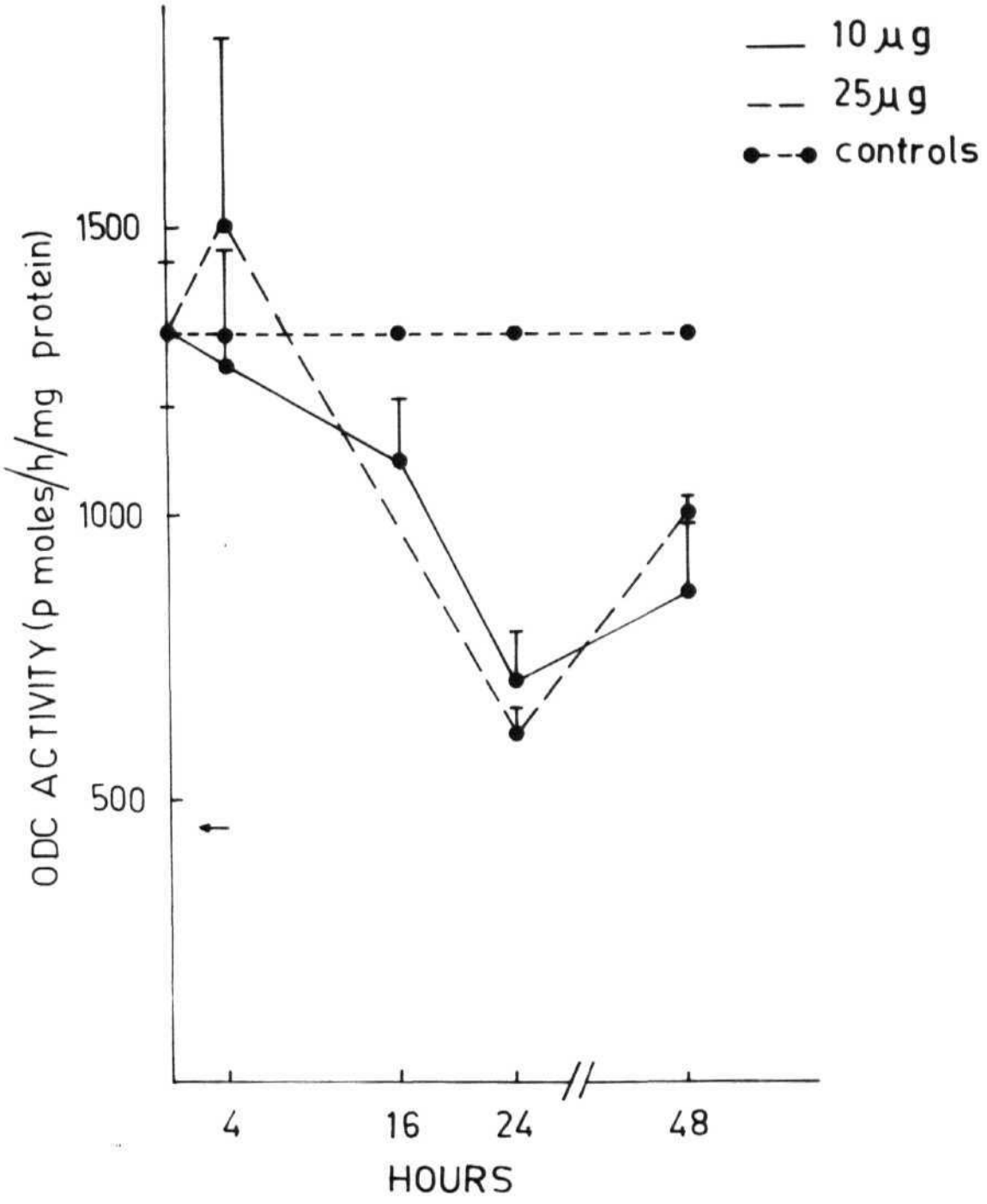

10 µg
25µg
controls
1500
1000
500
ODC ACTIVITY (p moles/h/mg protein)
4
16
24
48
HOURS

Fig. 2 Dose response relationship for PGE_2-induced desensitization of testicular ODC. Testes were injected with 10, 25 or 50µg of PGE_2 and at 24h thereafter the animals were given a second intra-testicular injection of PGE_2 (10µg/testis). ODC activity was estimated at 2h after the second injection. S-Represents injection of 10µl of saline-ethanol mixture (9:1, v/v) as vehicle. a, b and c - Significantly different compared to the group injected with vehicle followed by PGE_2 (10µg) ($p<0.001$) for 2h. Results are mean ± S.E. M. of 3-5 determinations from 6-10 animals in each group.

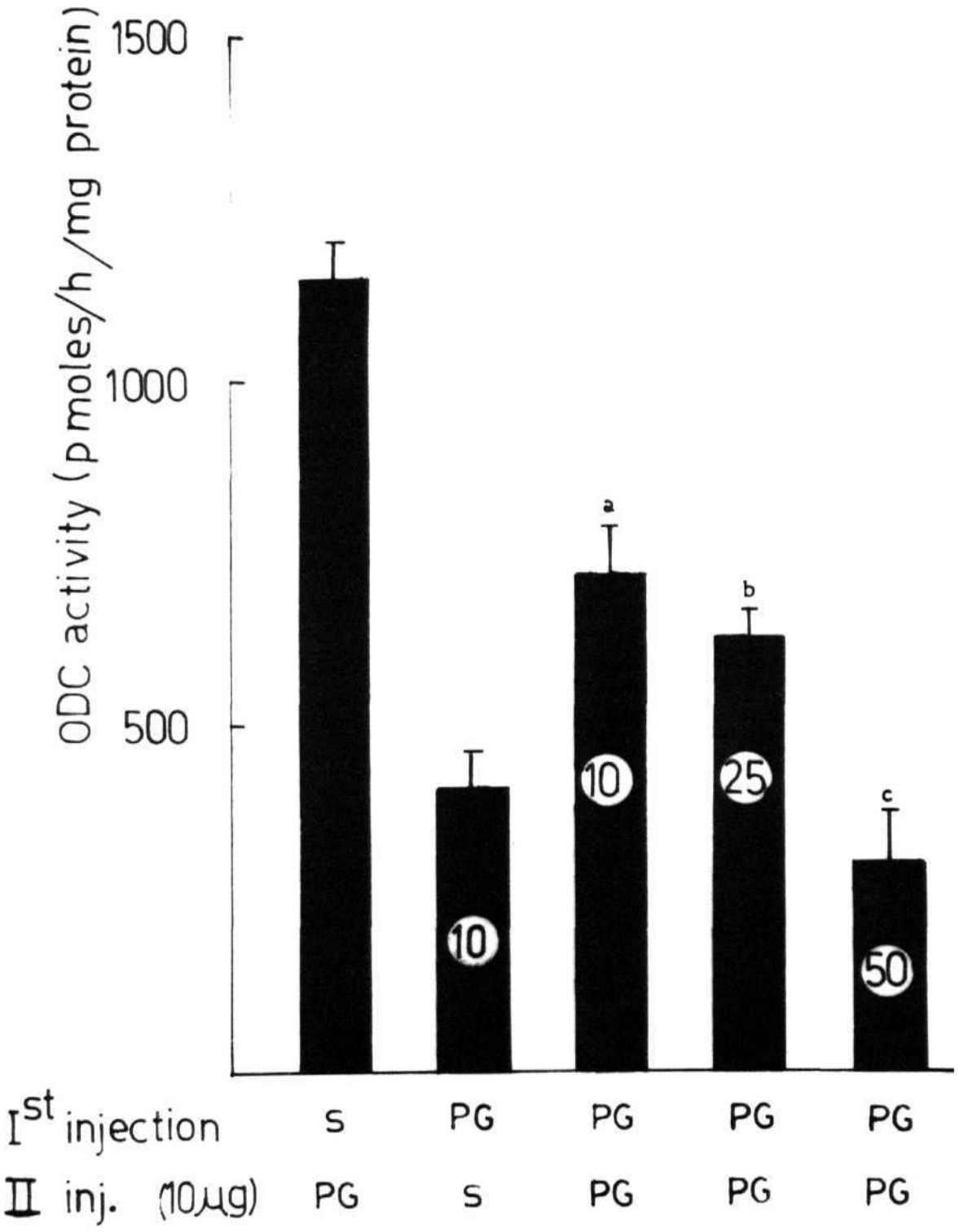

ODC activity (p moles/h/mg protein)
1500
1000
500
a
b
c
10
10
25
50
I st injection
II inj. (10µg)
S
PG
S
PG
PG
PG
PG
PG
PG
PG

Fig. 3 Effect of $PGF_{2\alpha}$ on desensitization of testicular ODC. $PGF_{2\alpha}$ at a dose of 10µg or 25µg per testis was injected intratesticularly. At 24h thereafter 10µg of $PGF_{2\alpha}$ was injected and OX levels were estimated after an interval of 2h. S-Represents injection of 10µl saline-ethanol (9:1, v/v) as vehicle. a-Significantly different ($p<0.02$) compared with the group treated with vehicle followed by $PGF_{2\alpha}$ (10µg) for 2h. Results are mean ± S.E.M. of 3-5 determinations from 6-10 animals in each group.

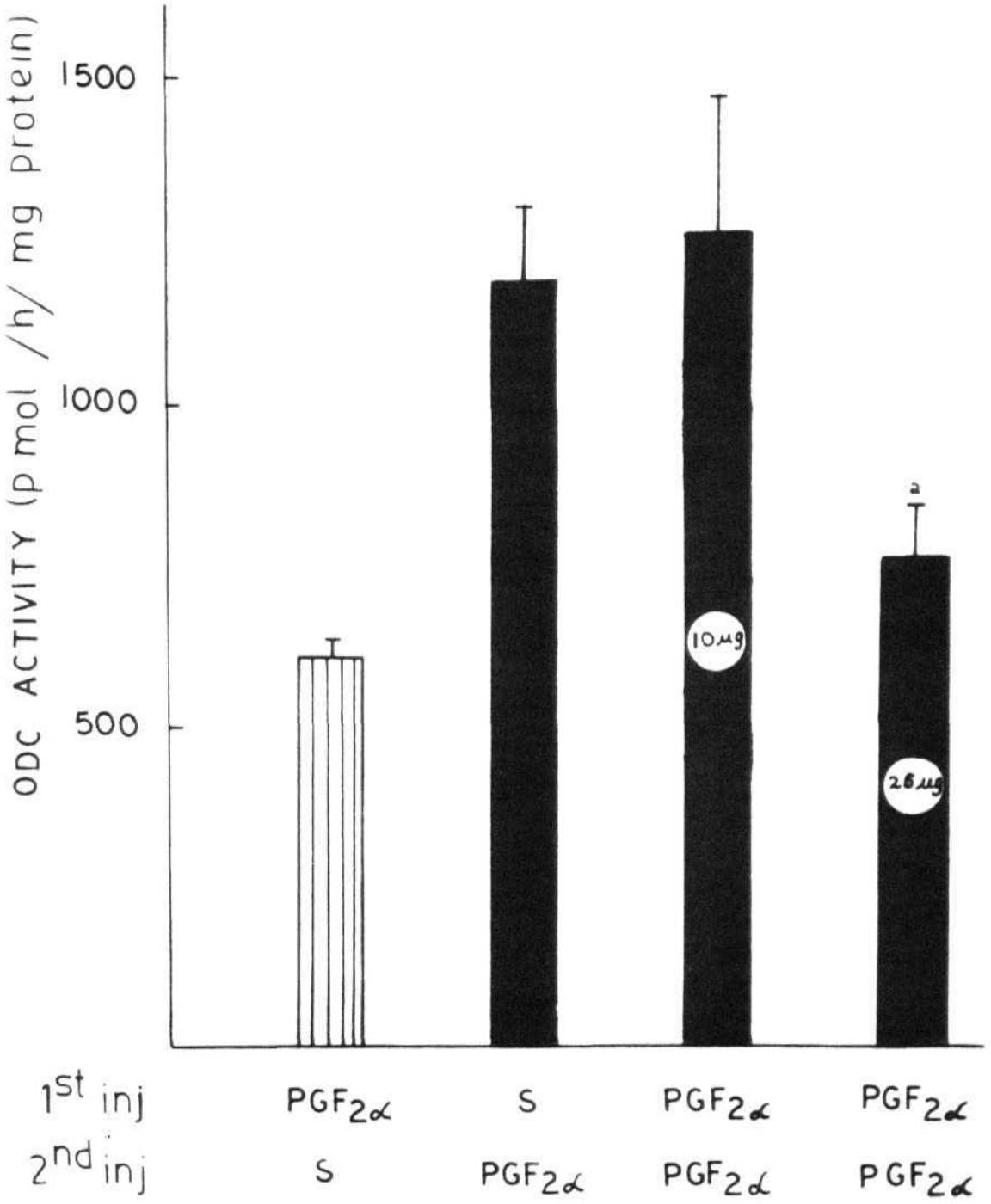

ODC ACTIVITY (p mol /h/ mg protein)
1500
1000
500
a
10μg
2.6μg
1st inj
2nd inj
PGF2α
S
S
PGF2α
PGF2α
PGF2α
PGF2α
PGF2α

Fig. 4 Effect of FSH, LH and cAMP on PGE_2 desensitized testis. At 24h after the injection of 10µg of PGE_2, 40µg of LH, 40µg of FSH, 20µg of cAMP or 10µg of PGE_2 were injected and the animals were killed 2h later for the estimation of ODC activity in the total testis. a, b, c and d - Significantly different when compared with the respective controls injected with similar doses of PGE_2, LH, FSH or cAMP alone ($p<0.01-0.001$). Results are mean ± S.E.M. of 3-5 determinations from 6-10 animals in each group. S-Represents injection of 10µl saline-ethanol mixture (9:1, v/v) as vehicle.

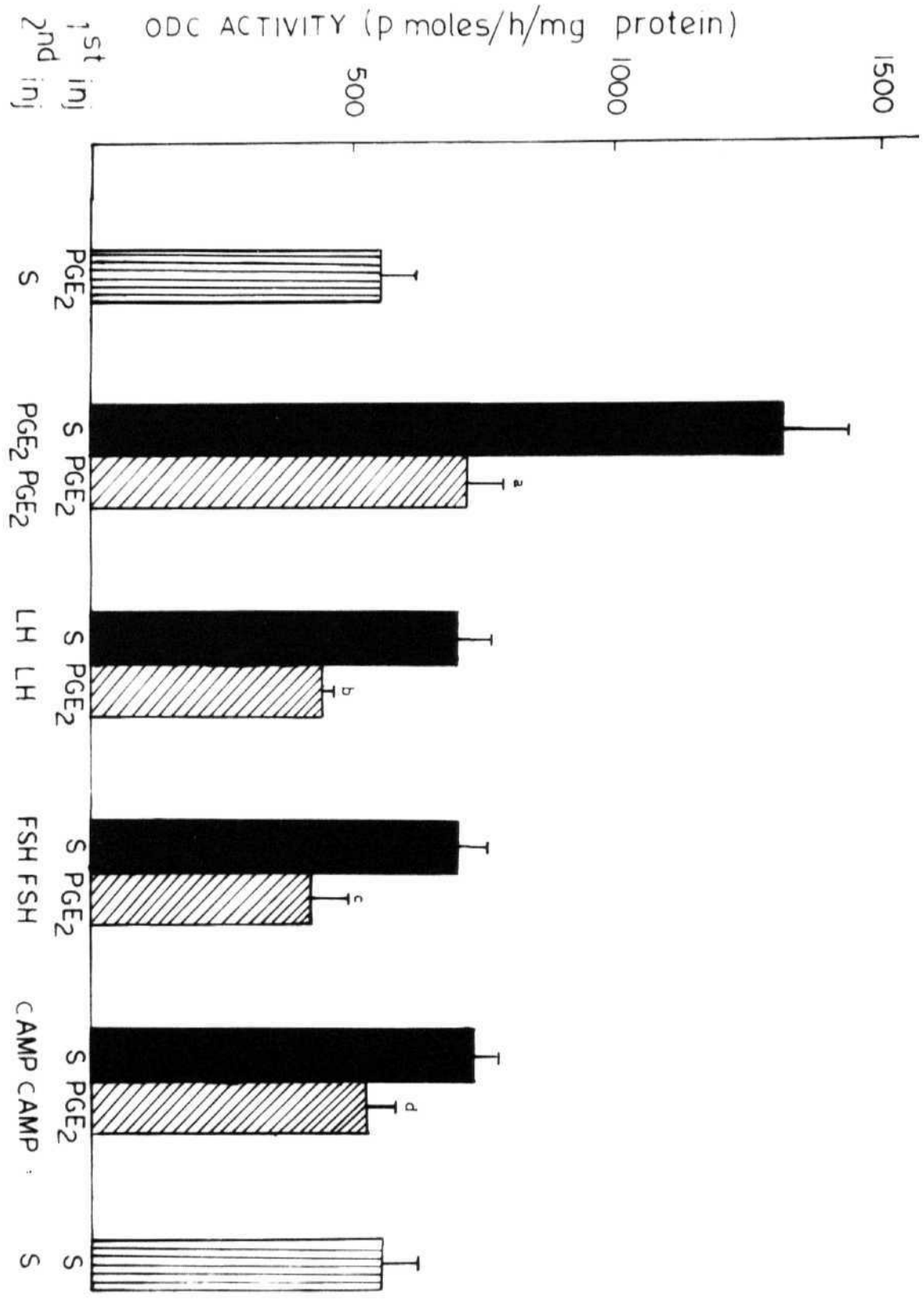

ODC ACTIVITY (p moles/h/mg protein)
500
1000
1500
1st inj
2nd inj
S
PGE2
S PGE2 PGE2
LH
S PGE2 LH
FSH
S PGE2 FSH
CAMP
S PGE2 CAMP
S
a
b
c
d

Fig. 5 Effect of PGE_2, FSH, LH and cAMP on Leydig cell and seminiferous tubule fractions in the PGE_2 desensitized testis. The testis was desensitized with 10µg of PGE_2. At 24h after the first injection of PGE_2 a second injection of 10µg of PGE_2, 40µg of FSH, 40µg of LH or 20µg of cAMP were given and the activity of ODC estimated in the Leydig cell and seminiferous tubule fractions at 2h. a, b, c and d - Significantly different from the corresponding controls injected with similar doses of PGE_2, LH, FSH or cAMP alone ($p<0.01-0.001$). Results are mean ± S.E.M. of 3-5 determinations from 6-10 animals in each group. S-represents injection of 10µl of saline-ethanol mixture (9:1, v/v) as vehicle.

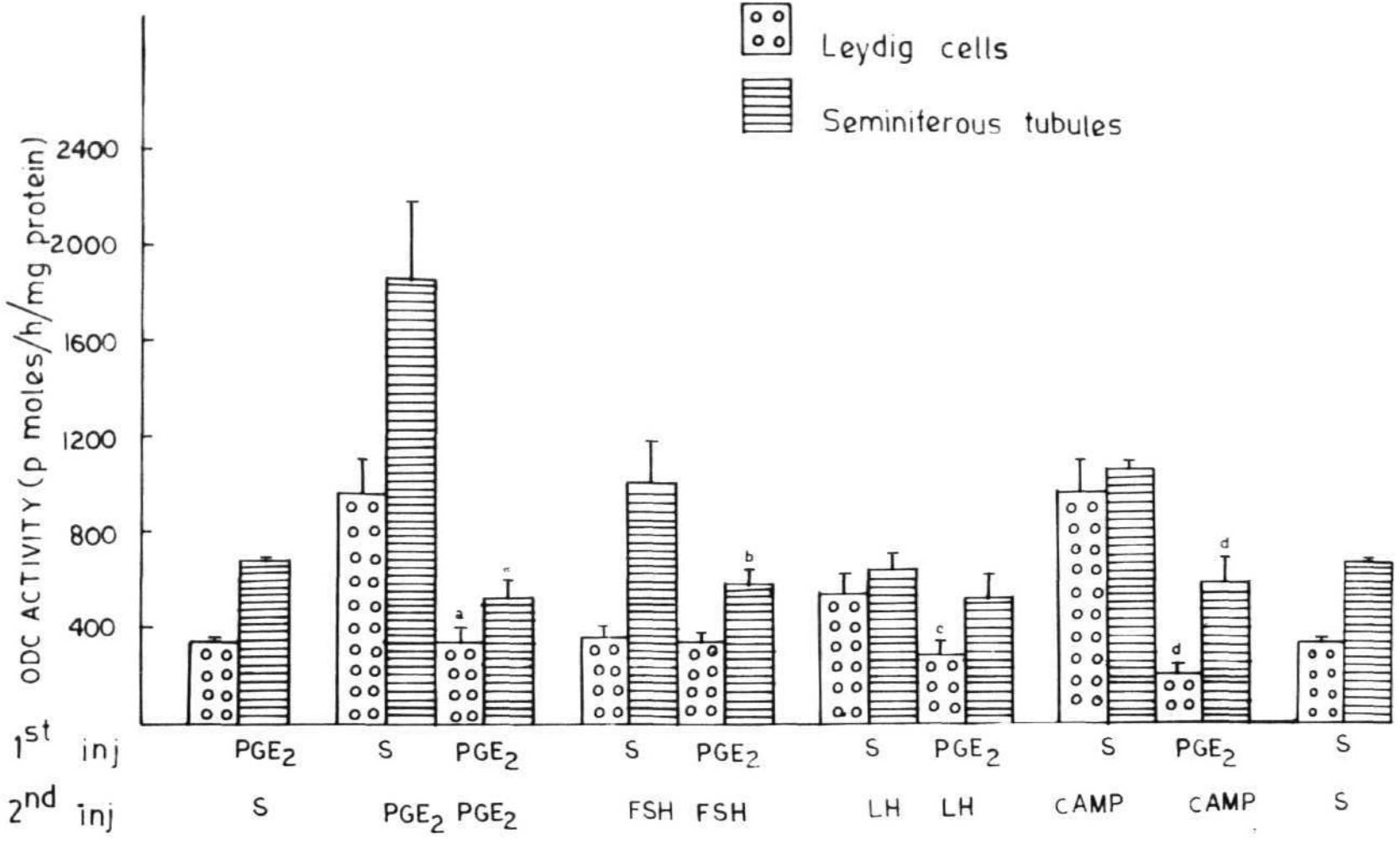
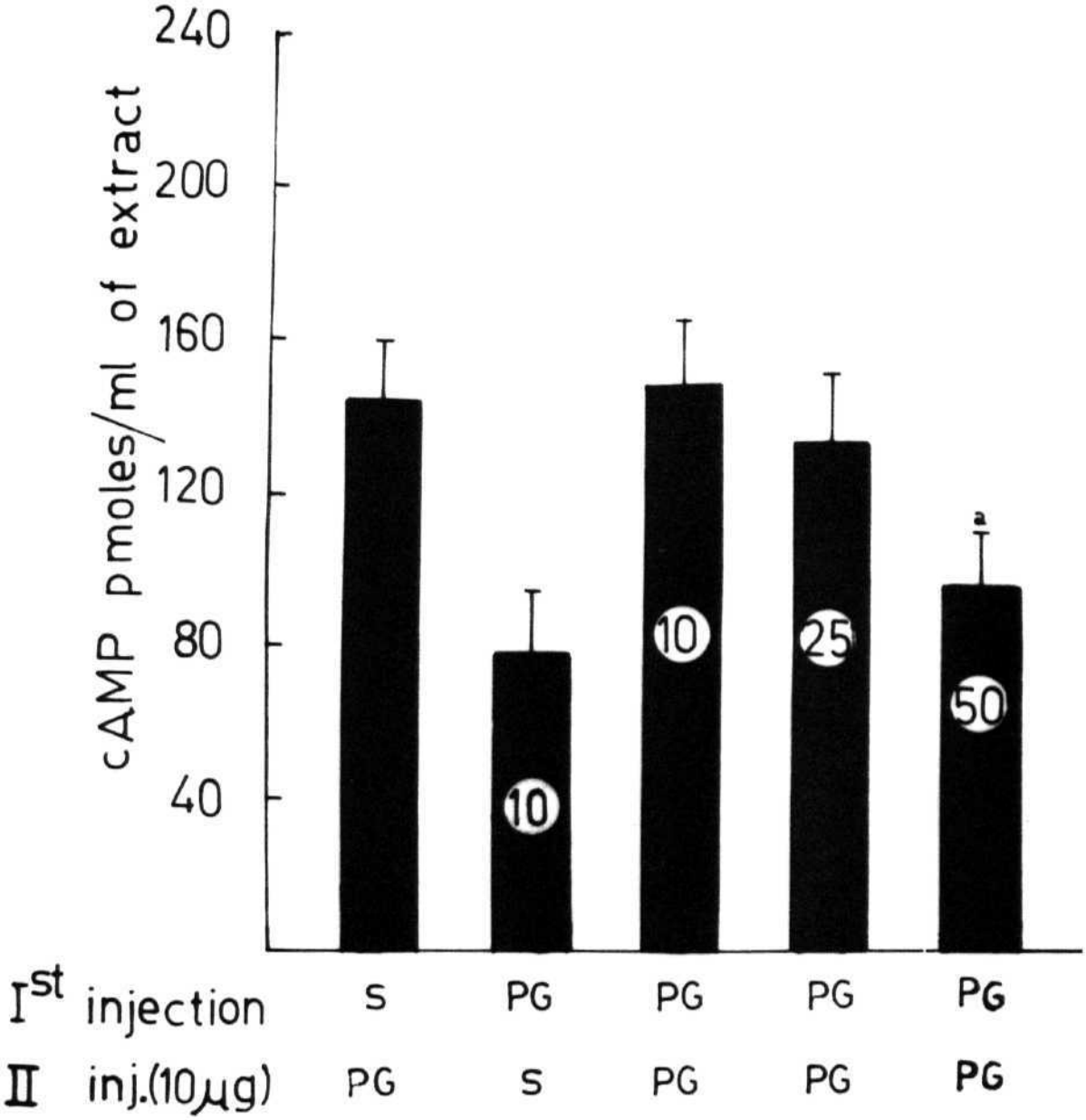

Leydig cells
Seminiferous tubules
ODC ACTIVITY (p moles/h/mg protein)
2400
2000
1600
1200
800
400
a
b
c
d
1st inj
PGE2
S
PGE2
S
PGE2
S
PGE2
S
PGE2
S
2nd inj
S
PGE2 PGE2
FSH FSH
LH LH
cAMP
cAMP
S

Fig. 6 Effect of various doses of $PGF_{2\alpha}$ on desensitization of testicular cAMP levels. Groups of animals were injected with 10, 25 or 50µg of $PGF_{2\alpha}$ per testis. At 24h thereafter the animals were given a second intratesticular injection of $PGF_{2\alpha}$ (10µg/testis). cAMP content was estimated 30 rain after the second injection. a - Significantly different (p=0.05) from the animals treated with vehicle followed by $PGF_{2\alpha}$ for 2h. Results are mean ± S.E.M. of 3-5 determinations from 6-10 animals in each group. S-Represents injection of 10µl of saline-ethanol mixture (9:1, v/v) as vehicle.


240
200
160
120
80
40
cAMP pmoles/ml of extract
10
10
25
50
a
I st injection
II inj.(10µg)
S
PG
PG
PG
PG
PG
S
PG
PG
PG

CHAPTER IV

A. EFFECT OF CATECHOLAMINES ON ORNITHINE DECARBOXYLASE ACTIVITY IN THE TESTIS OF IMMATURE RAT.

B. DESENSITIZATION OF TESTICULAR ORNITHINE DECARBOXYLASE ACTIVITY TO NOREPINEPHRINE

EFFECT OF CATECHOLAMINES ON ORHITHINE DECARBOXYLASE ACTIVITY IN THE TESTIS OF IMMATURE RAT

INTRODUCTION:

The testis of rat contains adrenergic nerve innervation in close proximity to testicular blood vessels (Norberg et al, 1967). Large doses of epinephrine and norepinephrine affect testicular vasoconstriction and blood flow (Free and Jafee, 1972b; Gomes and VanDemark, 1974). It was observed that the general metabolic activity of the testis is altered by the addition of epinephrine in vitro to the testicular tissue (Ewing et al, 1964). Catecholamines caused reduction in the level of testosterone (Damber and Janson, 1978). However, the levels of cAMP were found to increase following treatment with catecholamines *in* vitro (Heindel et al, 1981).

In this chapter data on the modulation of the activity of ornithine decarboxylase in the testis of immature rat by catecholamines is presented. In the previous sections it was shown that testicular ornithine decarboxylase is strikingly responsive to hormonal effectors such as LH, FSH and certain prostaglandins. The present study shows that in the testis ODC activity is regulated by catecholamines probably through the mediation of α-adrenergic receptors.

MATERIALS AND METHODS:

The source of chemicals is given in Chapter II. Immature male rats, aged 21-22 days were injected intratesticularly under mild ether anesthesia with hormones, PGE_2 or cAMP in 5-10µl of 0.15M sodium chloride, ethanol or ethanol saline mixture. At appropriate time the animals were killed by spinal dislocation and the decapsulated testes were homogenized in 4 volumes of homogenizing buffer (Chapter II). The supernatant was used for the assay of ODC activity as described in Chapter II. Leydig cells and seminiferous tubules were isolated by incubation in Krebs-Ringer bicarbonate buffer containing 1mg/ml collagenase as detailed in General Materials and Methods. Protein content was measured by the procedure of Lowry *et al* (1951).

RESULTS;

The effect of injection of epinephrine at a dose of 1µg/testis on total testicular ODC activity at various time intervals is given in Fig. 1. It was observed that maximal stimulation of ODC occurred at 2h after the injection of epinephrine. The enzyme activity declined at later time intervals and was comparable to control levels at 6h.

The effect of various doses of epinephrine and norepinephrine on ODC activity at 2h after the injection is given

in table 1. Both epinephrine and norepinephrine caused significant stimulation of the enzyme activity following injection with 100pg of the drug. Catecholamines did not cause any effect at lower than this dose. Maximal stimulation of the enzyme activity was observed with 500ng of epinephrine and 1µg of norepinephrine. The effect of various other neurotransmitters on ODC activity is given in table II. Isoproterenol (1µg/testes) caused significant stimulation of ODC activity at 2h after the treatment. Dopamine, tyramine and serotonin did not cause stimulation of the enzyme at a dose of 1µg/testis.

The effect of epinephrine and norepinephrine on Leydig cell and seminiferous tubule fractions is given in table III. Both epinephrine and norepinephrine caused significant stimulation of OX activity in the isolated Leydig cells and seminiferous tubule fractions at 2h after treatment. Table IV shows the effect of combined treatment with epinephrine and FSH, LH, PGE_2, norepinephrine or cAMP on ODC activity at 2h after the injection. It was observed that simultaneous injection of any of the above compounds to epinephrine treated animals caused significantly higher stimulation of the enzyme activity.

The effect of various α-and β-adrenergic antagonists on epinephrine stimulated OX activity is given in table V. The β-adrenergic antagonist propranolol and practolol did not inhibit epinephrine induced OX activity. D-L Propranolol

even at a high dose of 50µg/testis did not cause inhibition of epinephrine stimulated ODC activity. On the contrary 10µg of phenoxybenzamine, the α-adrenergic antagonist, caused significant inhibition of epinephrine stimulated ODC activity.

DISCUSSION:

The results presented in this chapter show that epinephrine, norepinephrine and isoproterenol cause stimulation of ODC activity. Both epinephrine and norepinephrine caused dose and time dependent stimulation of OX activity. Catecholamines increased the enzyme activity in both Leydig cells and seminiferous tubules.

It is interesting to note that injection of norepinephrine, PGE_2, LH, FSH or cAMP to animals treated with saturating dose of epinephrine cause additional stimulation of ODC activity. This may be due to the action of these compounds on different types of cells causing hypers timul at ion of ODC activity in the whole testis. Additional stimulation of ODC activity observed in this study following treatment with norepinephrine to epinephrine treated animals is interesting. This may be due to the action of these two catecholamines on two different types of receptors on the same cell or due to their action on entirely different population of cells in the testis. Furthermore, PGE_2 which also acts on both seminiferous tubules and Leydig cells also caused additional

stimulation of the enzyme in animals treated with saturating dose of epinephrine. These results support the hypothesis that hyperstimulation of ODC activity in these animals is due to the action of these different compounds on different types of cells.

The effect of epinephrine was not inhibited by the β-adrenergic receptor antagonists, propranolol and practolol, while it was blocked by α-adrenergic receptor antagonist, phenoxybenzamine. This indicates that the stimulation of ODC by catecholamines is mediated through functional α-adrenergic receptors in the testis.

It was proposed earlier (Chapter III A) that the increase in the levels of ODC following injection with LH, FSH, PGE_2 and $PGF_{2\alpha}$ is mediated through cAMP in the testis of rat. The mechanism of catecholamine stimulation of ODC in the testis is not clear. It is possible that cAMP is involved in the stimulation of ODC by catecholamines. Additional stimulation of ODC observed in this study in response to epinephrine and LH, FSH, PGE_2 or norepinephrine may be due to the increased stimulation of cAMP in response to the combined action of these agents. Heindel et al (1981), showed that stimulation of cAMP in rat Sertoli cells is prevented by β-adrenergic antagonists. In the present work it was observed that induction of ODC, on the other hand is prevented by prior treatment

with α-adrenergic blocking agents in the testis. This indicates that ODC induction in the rat testis is mediated through a mechanism involving a-adrenergic receptors.

TABLE I.

EFFECT OF VARIOUS DOSES OF EPINEPHRINE AND NOREPINE-PHRINE ON ODC ACTIVITY IN THE TESTIS

OX activity was measured at 2h after the injection of the compounds. The results are mean ± S.E.M. of 3-5 determinations from 6-10 animals per "group.

Treatment	Dose/Testis	OX activity (pmoles/h/mg protein)
Saline	-	709 ± 112
Norepinephrine	100 pg	1087 ± 51
	10 ng	1047 ± 33
	100 ng	1197 ± 94
	1.0 µg	1419 ± 19
	10.0 µg	1628 ± 140
Epinephrine	100 pg	1083 ± 43
	500 ng	1353 ± 113
	1.0 µg	1268 ± 114
	10.0 µg	1357 ± 121

All treated groups were statistically significant ($P<0.05$–$P<0.001$) when compared to the saline treated controls.

TABLE II

EFFECT OF VARIOUS NEUROTRANSMITTERS ON ODC ACTIVITY IN TESTIS

ODC activity was measured at 2h after the injection of the compounds. The results are mean ± S.E.M. of 3-5 determinations from 6-10 animals per group.

Group	Treatment	ODC activity (pmoles/h/mg protein)
1.	Saline	709 ± 64
2.	Epinephrine (1µg)	1268 ± 114
3.	Norepinephrine (1µg)	1419 ± 19
4.	Isoproterenol (1µg)	1313 ± 60
5.	Dopamine (1µg)	547 ± 59
6.	Tyramine (1µg)	611 ± 65
7.	Serotonin (1µg)	868 ± 75

Groups 2, 3 and 4 are significantly different ($P<0.001$) from group 1 controls.

TABLE III

EFFECT OF EPINEPHRINE AND NQREPINEPHRENE ON ODC ACTIVITY IN LEYDIG CELLS AND SEMINIFEROUS TUBULES

Leydig cell and seminiferous tubule fractions were separated as described in General Materials and Methods section. ODC activity was measured at 2h after the injection of catecholamines. Each group consists of 3-4 determinations containing 15-20 animals.

Treatment	ODC activity (pmoles/h/mg protein)	
	Leydig cells	Seminiferous tubules
Control	322 ± 18	549 ± 40
Epinephrine, 1µg	678 ± 101	999 ± 96
Norepinephrine, 1µg	754 ± 43	1064 ± 34

All treated values are significantly ($P<0.001$) different from controls.

TABLE IV

COMBINED EFFECT OF CATECHOLAMINES, GONADOTROPIC HORMONES, PGE_2 AND cAMP ON ODC ACTIVITY IN THE TESTIS

ODC activity was estimated at 2h after the injection of the various compounds. The results are mean ± S.E.M. of 3-5 determinations from 6-10 animals per group.

Group No.	Treatment	ODC activity (pmoles/h/mg protein)	Comments
1.	Control	709 ± 64	
2.	Epinephrine (1µg)	1268 ± 114	p<0.01 vs group 1
3.	Norepinephrine (1µg)	1419 ± 19	p<0.001 vs group 1
4.	Epinephrine (1µg) + Norepinephrine (1µg)	2328 ± 230	p<0.01 vs group 2
5.	Epinephrine (1µg) + cAMP (20µg)	1870 ± 143	p<0.05 vs group 2
6.	Epinephrine (1µg) + LH (40µg)	1772 ± 142	p<0.05 vs group 2
7.	Epinephrine (1µg) + FSH (40µg)	1992 ± 87	p<0.01 vs group 2
8.	Epinephrine (1µg) + PGE_2 (10µg)	2188 ± 106	p<0.001 vs group 2

TABLE V

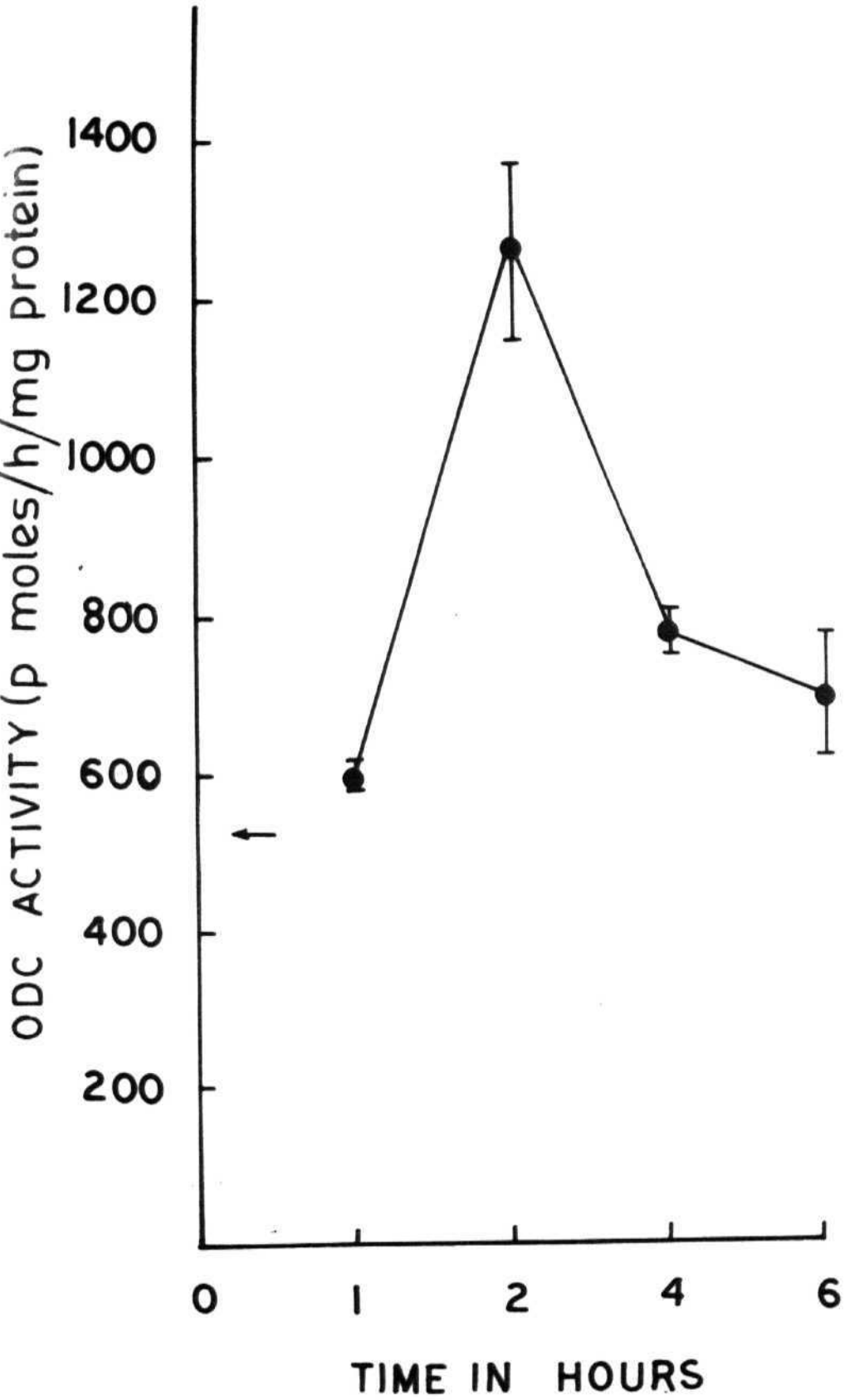
EFFECT OF α- AND β-ADRENERGIC ANTAGONISTS ON EPINEPHRINE STIMULATED ODC ACTIVITY

Epinephrine was injected in 10µl of saline. Propranolol practalol and phenoxybenzamine were injected intratesticularly 15 min before the injection of epinephrine in 5-10µl of salinet ethanol mixture (9:1, v/v). All animals were killed at 2h after the injection of epinephrine. Results are expressed as mean ± S.E.M. of 3-5 determinations from 6-10 animals per group.

Treatment	ODC activity (pmol/h/mg protein)
1. Control	709 ± 64
2. Epinephrine (1µg)	1716 ± 114
3. Epinephrine (1µg) + Propranolol (10µg)	1872 ± 60
4. Epinephrine (1µg) + Propranolol (50µg)	1651 ± 141
5. Epinephrine (1µg) + Practolol (10µg)	1530 ± 54
6. Epinephrine (1µg) + Phenoxybenzamine (10µg)	948 ± 53[a]
7. Epinephrine (1µg) + Phenoxybenzamine (20µg)	1108 ± 119[a]

a-significantly different ($p<0.01$) from group 2.

Fig. 1 Time course of action of epinephrine on ODC activity in the testis. Epinephrine was injected intratesticularly at a dose of 1µg/testis and the animals were killed at various time intervals. The arrow indicates the untreated control value. Results are man ± S.E.M. of 3–5 determinations from 6–10 animals in each group.


ODC ACTIVITY (p moles/h/mg protein)
1400
1200
1000
800
600
400
200
0
1
2
4
6
TIME IN HOURS

DESENSITIZATION OF TESTICULAR ORNITHINE DECARBOXYLASE ACTIVITY TO NOREPINEPHRINE

INTRODUCTION:

Exposure of target organ or cells to hormones may modify their response to subsequent stimulation by the same or by other hormones. Prolonged stimulation of the target tissue to hormones and catecholamines results in subsequent attenuation of the cyclic AMP response (Manganiello and Vaughan, 1972$ Franklin et al, 1975; Hsueh *et* al, 1977; Lamprecht et al, 1977; Terasaki et al, 1978; Perkins et al, 1978).

In the previous section it was reported that norepinephrine enhances ODC activity in the whole testis and in isolated Leydig cells and seminiferous tubules. In the present section evidence is presented to show that prior exposure to norepinephrine results in a dose and time dependent desensitization with respect to ODC activity.

MATERIALS AND METHODS:

The materials and animals were obtained as described previously in Chapter II. At appropriate time the decapsulated testes were homogenized in buffer (Chapter II). The homogenate was centrifuged at 25,000 x g for 30 minutes and the supernatant was used for the estimation of ODC activity as detailed in Chapter II.

RESULTS:

Fig. 1 shows norepinephrine induced desensitization as a function of time. Animals were treated intratestlcularly with 10µg of norepinephrine per testis and at an appropriate time second injection of 1µg of norepinephrine was similarly administered. ODC activity was estimated at 2h after the second injection. The results show that 16h of pretreatment with norepinephrine caused partial desensitization. Maximal desensitization of the testis occurred by 24h pretreatment with norepinephrine. At 48h after the first injection with norepinephrine the testls was no longer desensitized as the second injection caused stimulation of ODC activity.

Table 1 shows the dose response relationship for norepine-phrine induced desensitization of testicular ODC. It was observed that desensitization occurs more slowly when lower concentrations of the agonist are used. Norepinephrine at a dose of 0.1µg/testis did not cause refractoriness of testicular ODC. However, at a dose of 1.0µg/testis it caused partial refractoriness. A dose of 10µg per testis caused pronounced desensitization. The higher dose of 50µg of norepinephrine was found to be toxic to the animals.

Fig. 2 shows the effect of LH, FSH, $PGF_{2\alpha}$, epinephrine and cAMP on the norepinephrine desensitized testis. In these experiments the testes were desentized by injecting

intratesticularly with 10µg of norepinephrine. After an interval of 24h a second injection of a stimulatory dose of one of the above compounds was given. The animals were killed at 2h after the second injection and the ODC activity of the decapsulated testes was estimated. The results show that desensitization with norepinephrine did not cause the testes refractory to any of these compounds.

Fig. 3 shows the effect of norepinephrine on $PGF_{2\alpha}$ desensitized testis. In the preceding chapter it was shown that injection of $PGF_{2\alpha}$ at a dose of 25µg per testis caused refractoriness in the testis as the second injection of stimulatory dose of 10µg of $PGF_{2\alpha}$ at 24h after the first injection did not cause stimulation of ODC activity. In this study it was observed that injection of norepinephrine to $PGF_{2\alpha}$ desensitized testis caused significantly less stimulation of ODC activity when compared with norepinephrine alone treated animals.

DISCUSSION:

The present experiments demonstrate that after the first exposure to norepinephrine, the testis enters a period of refractoriness or desentiziation and during this time second injection with the stimulatory dose of norepinephrine does not cause stimulation of ODC. The extent of refractoriness depends both on the dose of the agonist and dur

of the first injection. Verhoeven et al. (1980) also observed that isoproterenol causes desensitization in Sertoli cells in vitro in terms of cAMP production and the desensitization was both dose and time dependent. In the present work it was observed that prior injection with norepinephrine does not cause the testis refractory to $PGF_{2\alpha}$, LH, FSH and cAMP. This type of desensitization which is specific to the homologous hormone is known as agonist specific desensitization (Plas and Nunez, 1975) Shear et al. 1976; Adachi et al, 1977; Lefkowitz and Williams, 1978).

The mechanism of desensitization process still remains to be elucidated. It has been reported that hormone induced refractoriness is accompanied by loss of membrane receptors or "down-regulation". Studies by Catt and his group show that the initial loss of measurable hCG receptors in the luteinized rat ovary is due to receptor occupancy where as secondary refractory state is due to the real loss of receptors (Catt et al, 1979b). In addition to "down regulation" of receptors other factors are also involved in the desensitization phenomenon (Johnson et al, 1978| Saez et al, 1979; Su et al, 1979). In this study it was shown that injection of $PGF_{2\alpha}$ results in marked cross-desensitization for norepinephrine where as norepinephrine desensitized testes are fully responsive to $PGF_{2\alpha}$. Probably these two compounds act through different membrane receptors due to the structural dissimilarities. Hence the sequence

of events leading to desensitization in response to $PGF_{2\alpha}$ and norepinephrine may be different, Harwood *et al*, (1979) observed that desensitization of ovarian luteal cells by hCG leads to the loss of responsiveness to both LH and epinephrine. Since the two hormones act via independent receptors to evoke cAMP production and steroidogenesis, these authors suggested that desensitization by hCG leads to a defect in the coupling of β-adrenergic receptors to adenylate cyclase.

Another interesting observation made in the present study is that norepinephrine desensitized testes are fully responsive to epinephrine. This could be due to the action of these compounds on two entirely different population of cells in the testis or due to their action on two different receptors on the same cell.

In another set of experiments it was observed that cAMP injection to norepinephrine desensitized testis caused stimulation of ODC activity in the whole testis; the GDC levels in the testis of these animals were comparable to the cAMP alone treated group. These results indicate that desensitization caused by norepinephrine is not due to a post cyclic AMP block while in the previous chapter (Chapter IIIB) it was observed that PGE_2 caused desensitization of the testis at a step beyond cAMP formation. Thus it appears that the mechanism of desensitization caused by prostaglandins and catecholamines in the testis is different in terms of ODC activity.

TABLE I

EFFECT OF VARIOUS DOSES OF NOREPINEPHRINE ON DESENSITIZATION OF ODC ACTIVITY IN THE TESTIS

24h after the first injection of graded doses of norepinephrine a second injection of stimulatory dose of norepinephrine (1μg) was injected. The animals were killed at 2h after the second injection and ODC levels were estimated in the testis. Results are mean ± S.E.M. of 3-5 determinations from 6-10 animals in each group.

	Treatment		ODC activity
	1st injection	2nd injection	(pmoles/h/mg protein)
1.	Saline	Saline	609 ± 20
2.	Saline	Norepinephrine	1419 ± 39
3.	Norepinephrine (0.1μg)	Norepinephrine (1.0μg)	1321 ± 210
4.	Norepinephrine (1.0μg)	Norepinephrine (1.0μg)	1039 ± 54[a]
5.	Norepinephrine (10.0μg)	Norepinephrine (1.0μg)	806 ± 62[b]

a–$p<0.01$ and b–$p<0.001$ when compared to group 2

Fig. 1 Effect of time on desensitization of ornithine decarboxylase response to norepinephrine in the testis. Following injection with 10μg of NE at 0 time a second injection of 1μg of NE was given at 4, 16, 24 or 48h. Controls (O———————0) were treated with vehicle alone at 0 time followed by a second injection with NE at 4, 16, 24 or 48h. Arrow represents ODC activity of animals treated with vehicle alone at 0 time and at 24h later. All animals were killed at 2h after the second injection of vehicle or NE for the assay of ODC activity. Values represent mean ± S.E.M. of 3-5 determinations from 6-10 animals in each group.

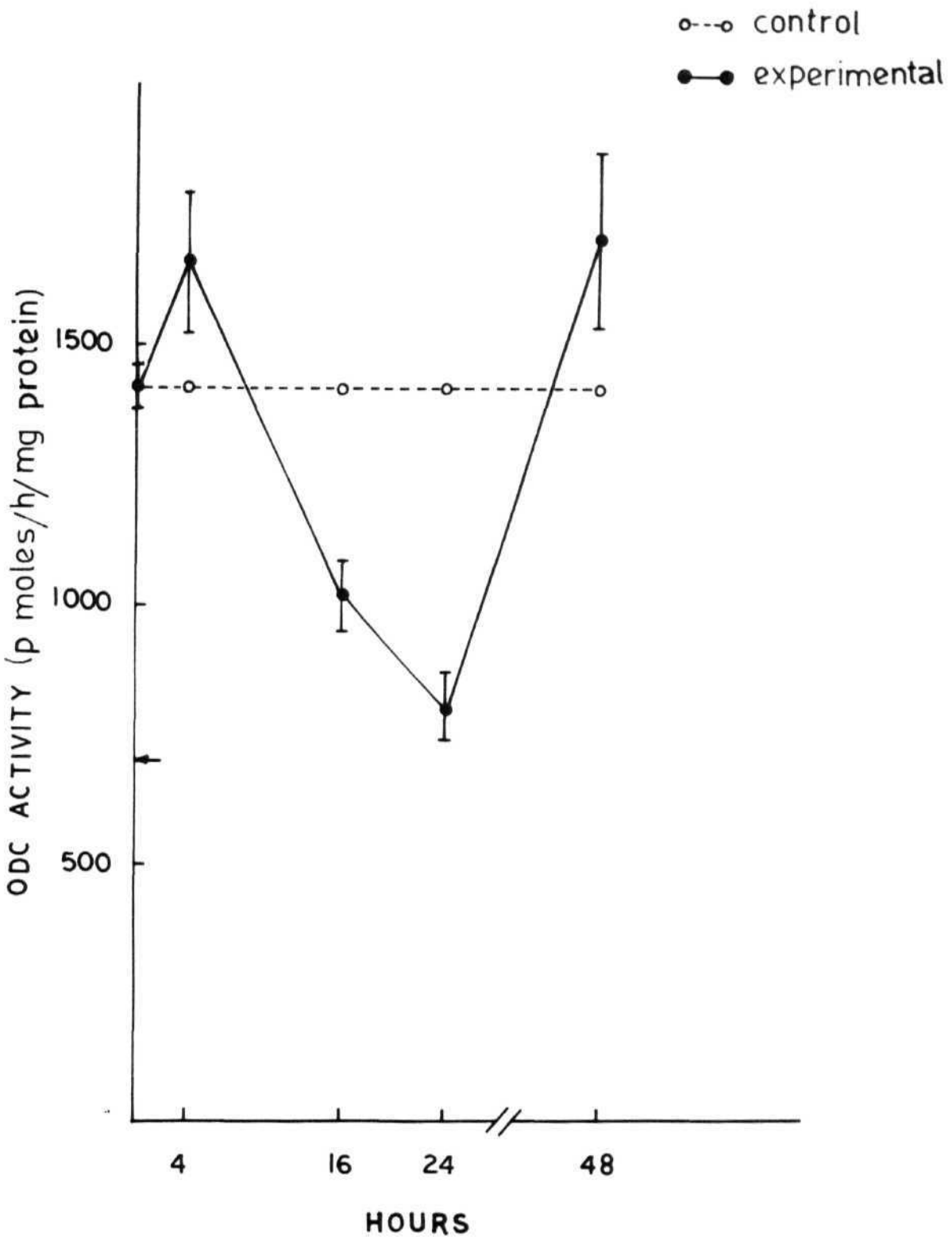

control
experimental
ODC ACTIVITY (p moles/h/mg protein)
1500
1000
500
4
16
24
48
HOURS

Fig. 2 Effect of FSH, LH, $PGF_{2\alpha}$ epinephrine and cAMP on norepinephrine desensitized testis. At 24h after the injection of 10µg of norepinephrine, 40µg of LH, 40µg of FSH, 1µg of epinephrine, 1µg of norepinephrine, 20µg of cAMP, 10µg of $PGF_{2\alpha}$ were injected intratesticularly and the animals were killed 2h later for the estimation of ODC activity in the testis. * - Significantly different ($p<0.001$) when compared to the group treated with vehicle followed by norepinephrine (1µg) for 2h. a, b, c, d and e - Statistically similar when compared with respective controls. Results are mean ± S.E.M. of 3-5 determinations from 6-10 animals in each group. S - Represents injection of 10µl of saline: ethanol mixture (9*1, v/v) as vehicle.

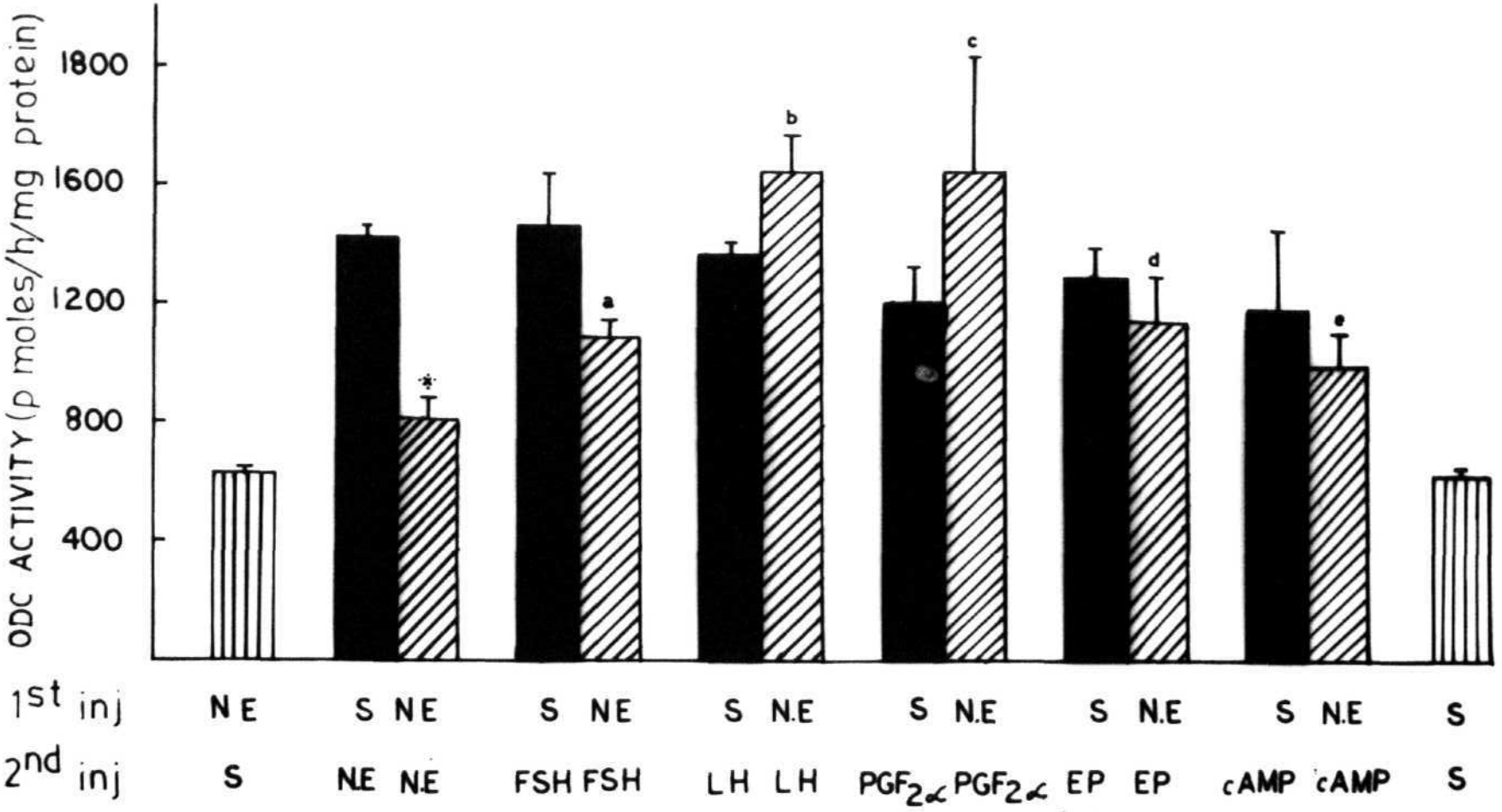
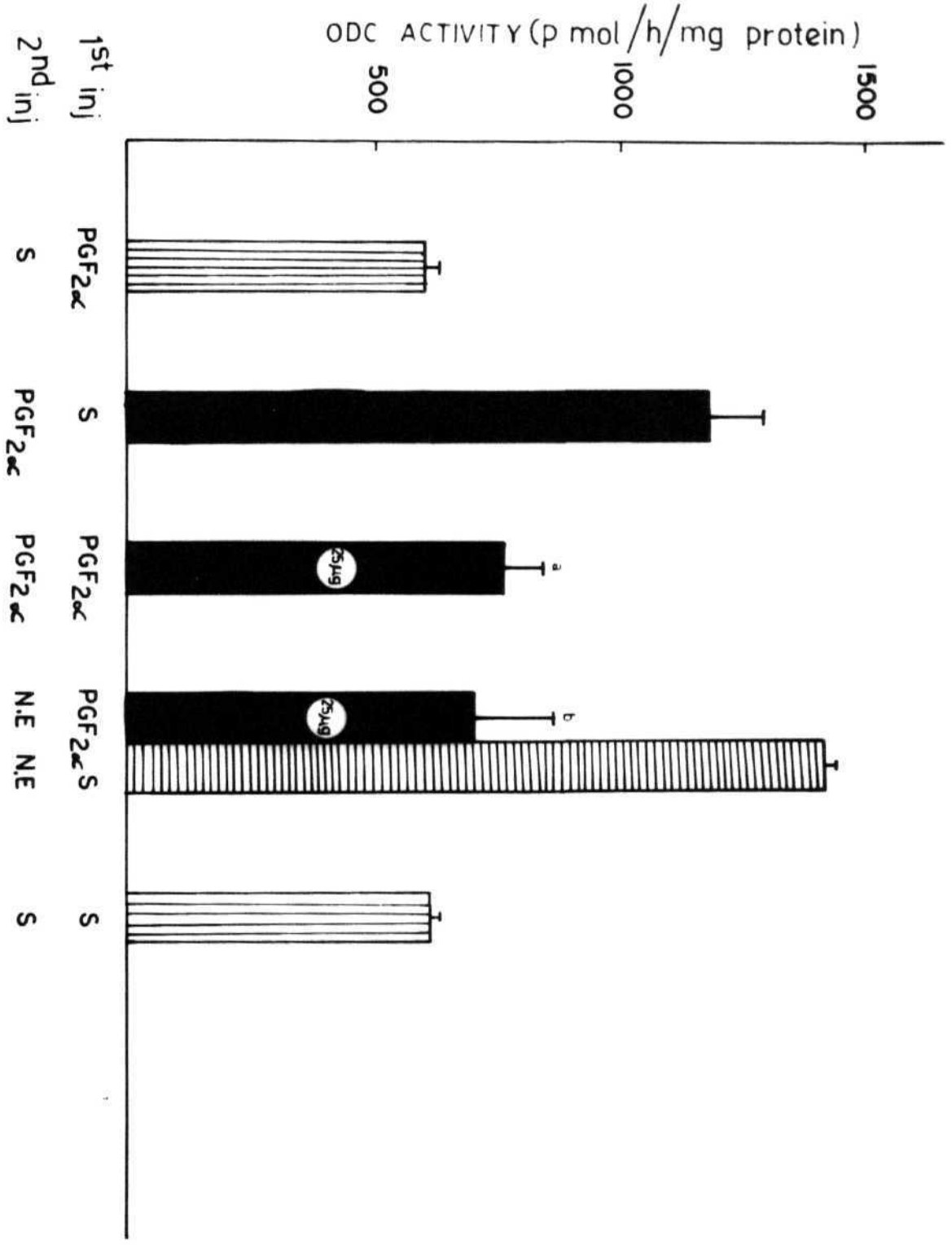

ODC ACTIVITY (p moles/h/mg protein)
1800
1600
1200
800
400
a
b
c
d
e
1st inj
2nd inj
N E
S
S NE
NE NE
S NE
FSH FSH
S N.E
LH LH
S N.E
PGF2α PGF2α
S N.E
EP EP
S N.E
cAMP cAMP
S
S

Fig. 3 Effect of norepinephrine on $PGF_{2\alpha}$ desensitized testis. Animals were intratesticularly injected with $PGF_{2\alpha}$ (25µg/testis) in the first injection. 24h thereafter norepinephrine (1µg/testis) or $PGF_{2\alpha}$ (10µg/testis) was given similarly and the animals were killed after an interval of 2h for the assay of CDC activity. a - Significantly different ($p<0.02$) when compared to animals treated with vehicle followed by $PGF_{2\alpha}$ (10µg/testis), b - Significantly different ($p<0.001$) compared to group treated with vehicle followed by norepinephrine (1µg/testis). S - Represents salinet ethanol mixture (9:1, v/v). Results are mean ± S.E.M. of 3-5 determinations from 6-10 animals in each group.


ODC ACTIVITY (p mol/h/mg protein)
500
1000
1500
1st inj
2nd inj
S
PGF2α
S
PGF2α
PGF2α
PGF2α
N.E
N.E
PGF2α
S
S
S
a
b

CHAPTER V

STIMULATION OF ORNITHINE DECARBOXYLASE ACTIVITY BY LUTEINIZING HORMONE RELEASING HORMONES IN THE TESTIS.

STIMULATION OF ORNITHINE DECARBOXYLASE ACTIVITY BY LUTEINIZING HORMONE RELEASING HORMONE IN THE TESTIS

INTRODUCTION:

Luteinizing hormone releasing hormone (LHRH) stimulates gonadotropic hormone production in the pituitary (Schally, 1978; Guillemin, 1978). However, paradoxically different effects of LHRH are also observed. LHRH and its potent synthetic LHRH analogs inhibit processes required for reproductive function in the female (Corbin et al, 1978) and in the male (Corbin et al, 1978| Rivier et al, 1979). These inhibitions are possibily due to imbalance in pituitary gonadotropin production in response to persistent stimulation of pituitary gland by LHRH (Belchetz et al, 1978) and/or due to the down regulation of gonadotropin receptors in target tissue by large amounts of gonadotropins released by the pituitary (Rivier et al, 1978; Catt et al, 1979b; Kledzik et al, 1978).

More recently, however, the ability of LHRH and its analogs to act directly on the male and female reproductive organs has been established. In the male, LHRH inhibits FSH-induced increase in testicular LH/hCG receptors and also inhibits testicular steroidogenesis in hypophysectomized male rats (Arimura et al, 1979; Hsueh and Erickson, 1979a). LHRH causes inhibition of steroid induced increase in the

weight of accessory reproductive organs of rat (Sundaram et *3k** **1981)** and causes inhibition of testosterone induced β-glucoronidase activity in mouse kidney (Lecomte et al, 1982). In the female LHRH inhibits FSH stimulated steroid production in vivo and in vitro (Hsueh and Erickson, 1979b; Hsueh et al, 1980; Massicotte et al, 1980; Knecht *et* al, 1981$ Jones and Hsueh, 1981| Gore-Langton et *al*, 1981), follicular development (Ying and Guillemin,1979) and FSH induced LH/hCG receptors in cultures of granulosa cells (Hsueh et al, 1980). LHRH agonists have also been found to inhibit FSH stimulated cAMP accumulation in granulosa cells (Massicotte et al, 1980; Knecht *et* al, 1981).

However, the effects of LHRH or LHRH analogs on granulosa cell function have varied from inhibitory to stimulatory depending upon the experimental conditions used and parameters examined. In a different series of experiments LHRH agonists were shown to stimulate prostaglandin accumulation (Clark et al, 1980; Clark, 1982), progesterone synthesis (Clark and Marsh, 1980; Knecht et al, 1981;Jones and Hsueh, 1981) and cause maturation of oocytes (Hillensjo and LeMaire, 1980). In view of these varying effects of LHRH, the present experiments were designed to study the effects of LHRH on the testis of immature rat using ODC as a parameter.

MATERIALS AND METHODS:

Immature male rats, derived from Wistar strain, aged 21-22 days were used in this study. LHRH was injected intratesticularly in a total volume of 5-10µl. At an appropriate time the rats were killed by spinal dislocation and the decapsulated testes were homogenized in 4 volumes of homogenizing buffer (Chapter II) and centrifuged at 25,000 x g for 30 min and the supernatant was used for the assay of ODC activity. Following treatment with LHRH Leydig cells and seminiferous tubules were separated by collagenase treatment and the ODC activity of the isolated cells was estimated as described in Chapter II. Protein content of the supernatant was determined according to the method of Lowry et al (1951).

RESULTS:

Fig. 1 shows the effect of intratesticular injection with 1µg of LHRH on ODC activity at various time intervals. As early as 1h after treatment, LHRH caused significant stimulation of ODC activity. Maximum stimulation was observed at 2h after treatment and declined at 4h and 6h.

The effect of various doses of LHRH on ODC activity at 2h after treatment is shown in Fig. 2. A minimum of 0.1µg of LHRH was necessary to cause stimulation of ODC

and maximal stimulation was observed with 1µg of the hormone. To assess the possibility that this effect of LHRH on OX is due to direct action on the testis or is due to the effect of LHRH on pituitary the animals were injected intraperitoneally with 1.0 or 10.0µg of LHRH in 0.1ml of saline. At 2h after the treatment the animals were killed and the testicular ODC activity was estimated. Table 1 shows that both of these groups showed no effect on ODC activity of the testis.

Table II shows the combined effect of LHRH and $PGF_{2\alpha}$, LH, or cAMP. Maximal effective dose of LHRH in combination with $PGF_{2\alpha}$, LH or cAMP caused additional stimulation of ODC activity over LHRH injected animals.

The effect of LHRH on isolated Leydig cells and seminiferous tubule fractions is given in Table III, Injection of LHRH at a dose of 1µg/testis resulted in significant stimulation of ODC activity in Leydig cell fraction while there was no effect on seminiferous tubules.

The phenomenon of LHRH desensitization in the testis is shown in Fig, 3. LHRH at a dose of 1µg/testis caused partial refractoriness at 24h to renewed exposure to the same hormone. LHRH at a dose of 10µg/testis caused further desensitization as a second injection of LHRH elicited

dimished response. The effect of LH, FSH, $PGF_{2\alpha}$, cAMP and norepinephrine on LHRH desensitized testis is shown in Fig. 3. De sensitization with 10µg of LHRH did not cause the testis refractory to any of these compounds at 24h after first injection of LHRH.

DISCUSSION;

These experiments show that LHRH causes stimulation of ODC activity in the testis of rat. Though intact non-hypophysectomised animals were used in this study the action of LHRH on the testis appears to be direct and is not mediated through the pituitary due to the following observations i) Intraperitoneal injection of 10µg of LHRH did not cause stimulation of ODC activity while intratesticular injection of a much smaller dose of 1µg caused maximal response in the testis 2) LHRH is known to cause release of both LH and FSH from the pituitary (Schally, 1978; Guillemin, 1978). However, intratesticular injection of LHRH caused stimulation of ODC activity only in the Leydig cells and there was no response in the seminiferous tubules. This indicates that LHRH injection has probably not caused the release of pituitary gonadotropic hormones. 3) Reddy and Villee(1975a) have shown that optimal stimulation of ODC activity occurs at 4h in response to intratesticular injection of gonadotropic hormones while in this study it was observed that LHRH causes optimal effect at 2h. 4) The results presented in

chapter VI show that LH and FSH induced CDC activity is inhibited by prior treatment with phenoxybenzamine while similar inhibition was not observed in LHRH injected animals. 5) Reeves et al (1980) have shown specific binding sites for LHRH in the testis of rat.

The stimulatory action of LHRH observed in this study differs from that of various other investigators who showed inhibitory effects of LHRH on various model systems (Rippel and Johnson, 1976} Hsueh and Erickson, 1979a,b; Arimura et al, 1979; Hsueh and Ling, 1979; Hsueh *et* al, 1980; Massicotte et al, 1980; Sundaram et al, 1981). On the contrary, these results support the stimulatory effects of LHRH. Recently LHRH agonists were found to increase morphological oocyte maturation (Hsueh et al, 1980; Hillensjo and LeMaire, 1980) and cause stimulation of prostaglandin accumulation (Clark et al, 1980). Similarly accumulation of progesterone in granulosa cell cultures was also observed in response to LHRH (Clark and Marsh, 1980; Clark, 1982).

The stimulatory effect of LHRH on testicular CDC activity was confined to Leydig cells. This could be due to the presence of LHRH receptors only on the Leydig cells. Reeves et al (1980) showed specific binding sites for radioiodinated LHRH on *the* Leydig cells and they could not observe any binding of LHRH in the seminiferous tubules. Gore-Langton et al (1981) also observed that LHRH had no effect on the

Sertoli cells of rat. The observations made in this study and by others support the view that the direct action of LHRH on testis is confined to Leydig cells alone.

Additional stimulation of ODC observed in this study in response to the injection of LHRH and cAMP or $PGF_{2\alpha}$ could be due to the stimulation of seminiferous tubules by cAMP and $PGF_{2\alpha}$. However, LHRH in combination with LH also caused additional stimulation. Since both of these hormones were shown to act on the Leydig cells alone these results are intriguing. It is possible that LHRH and LH act on independent receptors on the Leydig cells and cause additional stimulation through two different mechanisms.

It was interesting to note that prior injection with LHRH caused the testis refractory at 24h as second injection of stimulatory dose of LHRH did not cause an increase in ODC activity. It was also found that the phenomenon of desensitization with LHRH was dose dependent as higher amount of LHRH caused more profound desensitization. However, desensitization to LHRH was specific to LHRH alone as other ODC stimulating agents like LH, FSH, $PGF_{2\alpha}$, norepinephrine and cAMP stimulated the ODC activity in the LHRH desensitized testis. This type of desensitization is known as homologous desensitization and is widely reported in the literature for various hormones (Catt *et al*, 1979a).

The mechanism of action of LHRH in causing stimulation of

ODC is not clear. LHRH was shown to increase cAMP levels in pituitary *in vitro* (Borgeat et al, 1972). However, the involvement of cAMP in the mediation of LHRH action in the pituitary has been disproved (Naor et al, 1978; Conn et al, 1979; Sen and Menon, 1979). On the contrary LHRH and its agonists were shown to inhibit FSH induced cAMP accumulation in granulosa cells (Massicotte et al, 1980| Knecht, 1981). Recently Knecht and Catt (1981) showed that the agonists of LHRH cause inhibition of adenyl cyclase and stimulate phospho-diesterase activity in granulosa cells. This indicates that the inhibitory effects of LHRH are due to the combined effect of decreased production and increased degradation of cAMP. While the inhibitory effect of LHRH appears to be due to the decrease in the levels of cAMP the stimulatory effects of LHRH are associated with increased levels of prostaglandins. Clark et al (1980) and Clark (1982) showed that LHRH and its agonists cause accumulation of prostaglandins in the cultures of rat granulosa cells *in vitro*. This may adduce probable involvement of prostaglandins in LHRH action in inducing ODC activity as it was shown earlier that prostaglandins cause stimulation of ODC activity in the testis of rat (Chapter IV). However, the experiments reported in the following chapter (Chapter VI) rule out this possibility since preinjection of aspirin, a prostaglandin synthetase inhibitor, did not cause inhibition of LHRH induced ODC activity. In view of these results it appears that the mechanism of action of LHRH is complicated.

TABLE I

EFFECT OF INTRAPERITONEAL INJECTION OF LHRH ON ODC ACTIVITY OF TESTIS

1 or 10μg of LHRH was injected intraperitoneally in 21-22 day old rats in a total volume of 0.1ml. At 2h after the injection the animals were killed and ODC activity was measured. Results are mean ± S.E.M. of 3-5 determinations from 6-10 animals per group.

Treatment	ODC activity (pmoles/h/mg protein)
1. Saline	637 ± 115
2. LHRH (1μg)	677 ± 91
3. LHRH (10μg)	713 ± 46

TABLE II

EFFECT OF LHRH IN COMBINATION WITH cAMP $PGF_{2\alpha}$ OR LH ON ODC ACTIVITY IN THE TESTIS

All compounds were injected in 5–10µl of saline and at 2h ODC activity was estimated. The results are mean ± S.E.M. of 3-5 determinations from 6–10 animals in each group.

Treatment	ODC activity (pmoles/h/mg/protein)	Comments
1. Saline	609 ± 20	
2. LHRH (1.0µg)	1260 ± 85	$p<0.01$ vs group 1
3. LHRH (1.0µg)+cAMP (20µg)	1714 ± 46	$p<0.01$ vs group 2
4. LHRH (1.0µg)+LH (40µg)	1675 ± 66	$p<0.02$ vs group 2
5. LHRH (1.0µg)+$PGF_{2\alpha}$ (10.0µg)	2427 ± 106	$p<0.001$ vs group 2

TABLE III

EFFECT OF LHRH ON ODC ACTIVITY IN THE LEYDIG CELLS AND SEMINIFEROUS TUBULES

At 2h after the injection of 1µg of LHRH the animals were killed and the seminiferous tubules and Leydig cells were separated as described and the OX activity was estimated in the separated fractions. The results are Mean + S.E.M. of 3-4 determinations from 15-20 animals per groupT

Treatment	ODC activity (pmoles/h/mg protein)	
	Leydig cells	Seminiferous tubules
Control	293 ± 9	559 ± 32
LHRH (1µg)	435 ± 37*	628 ± 30

*p<O.Ol compared to control.

Fig. 1 Effect of intratesticular injection with LHRH on ODC activity at various time intervals. The arrow shows saline treated control value at 2h. LHRH was injected at a dose of 1μg/testis. Each point represent mean ± 3.E.M. of 3-5 determinations from 6-10 animals.

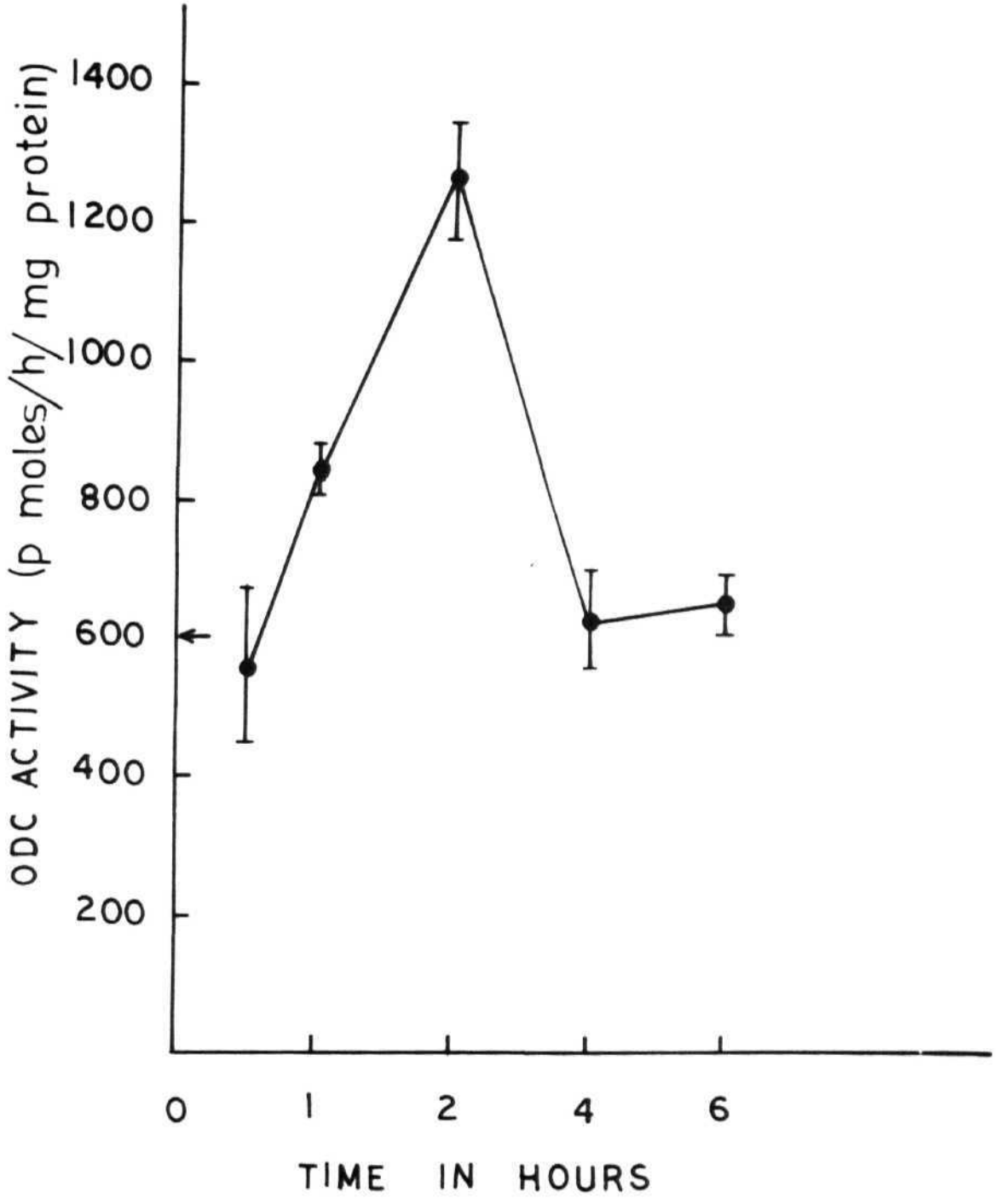

ODC ACTIVITY (p moles/h/ mg protein)
1400
1200
1000
800
600
400
200
0
1
2
4
6
TIME IN HOURS

Fig. 2 Effect of various doses of LHRH on ODC activity of immature rat testis. LHRH was injected intratesticularly and the enzyme activity was determined at 2h as described in materials and methods section. Each point is a mean ± S.E.M. of 3-5 determinations from 6-10 animals.

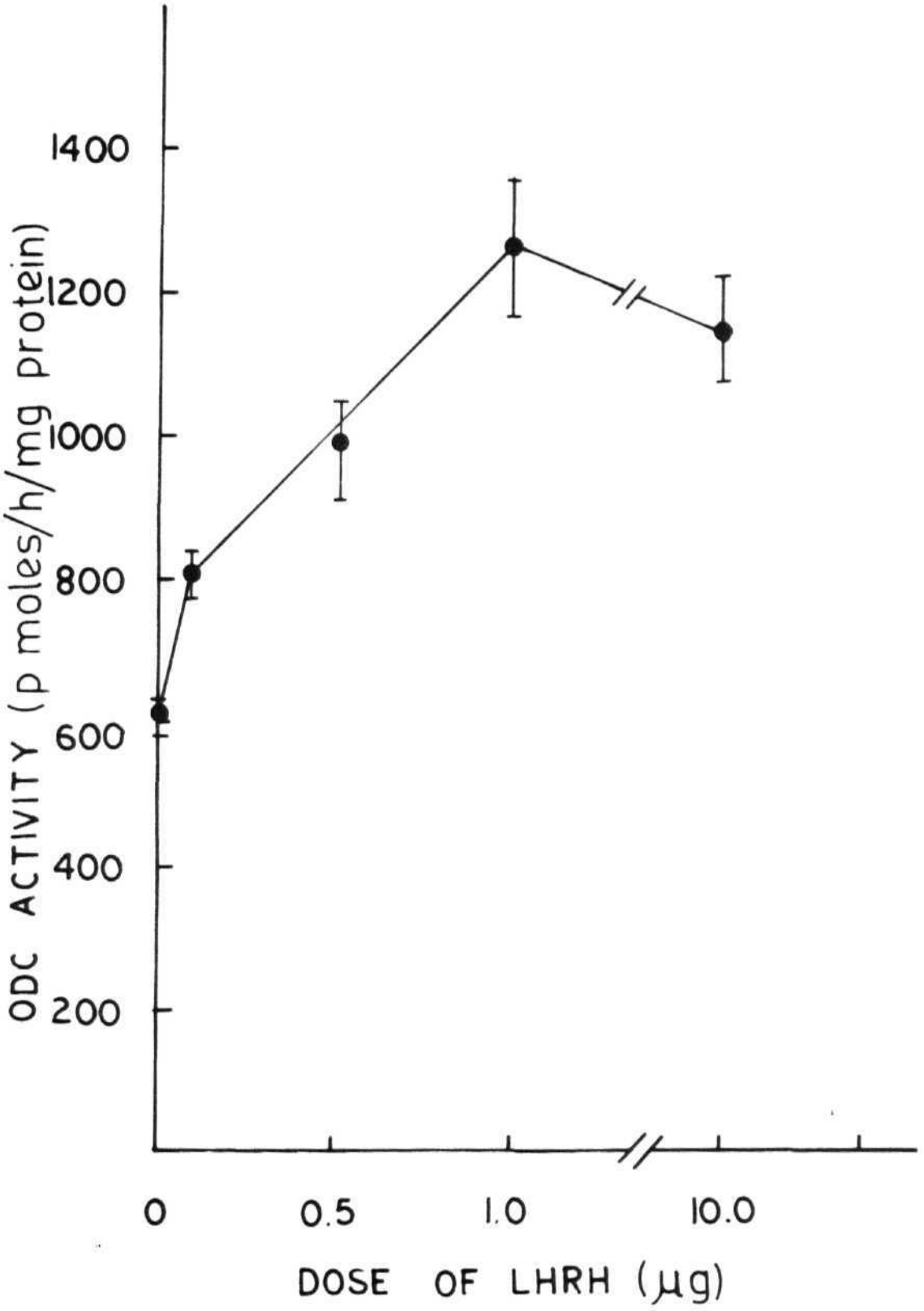
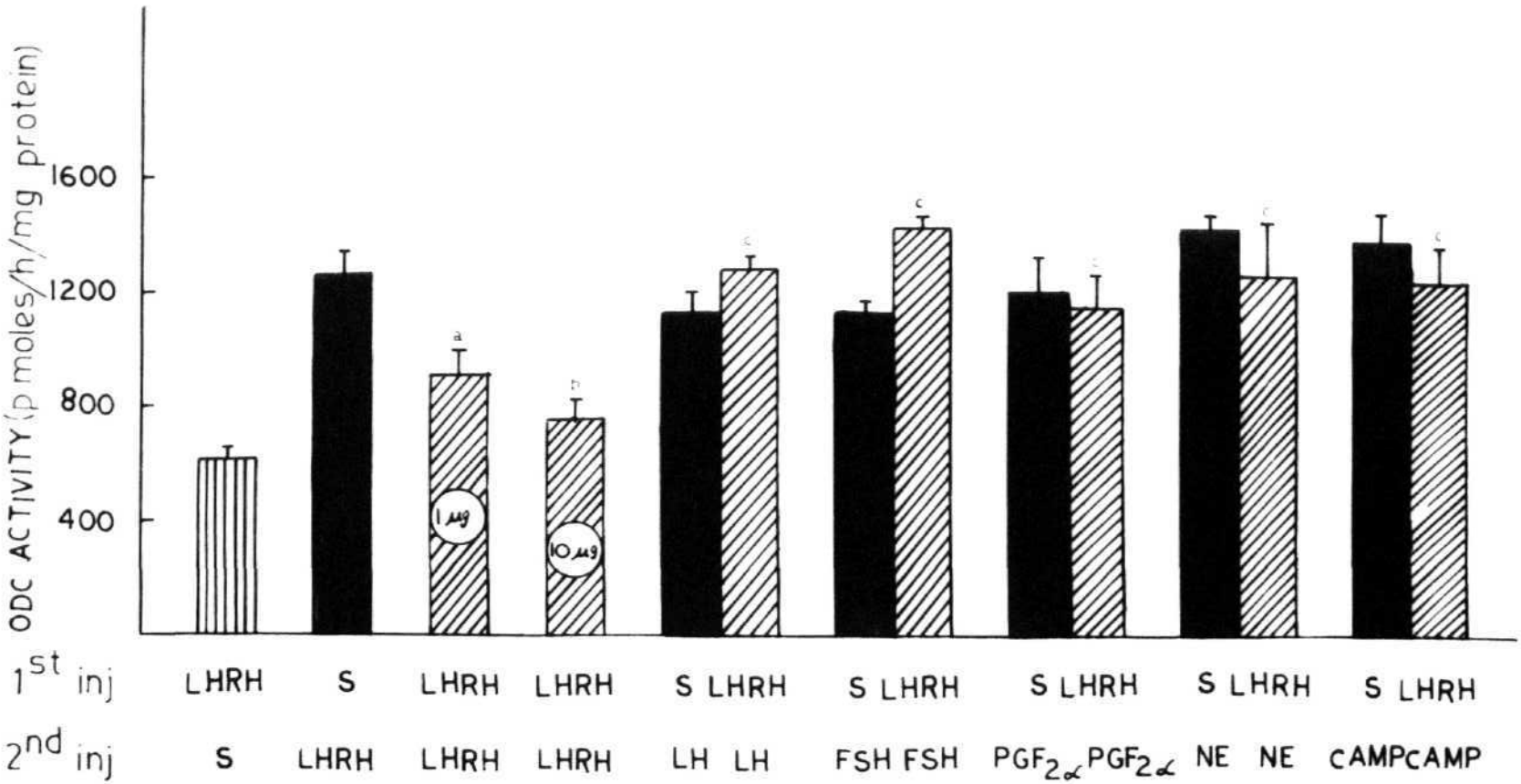

1400
1200
1000
800
600
400
200
ODC ACTIVITY (p moles/h/mg protein)
0
0.5
1.0
10.0
DOSE OF LHRH (µg)

Fig. 3 Effect of LH, FSH, $PGF_{2\alpha}$, norepinephrine and cAMP on LHRH desensitized testis. At 24h after the injection of 1 or 10µg of LHRH, 1µg of LHRH was injected and ODC levels monitered after an interval of 2h. Prior treatment with 1µg or 10µg of LHRH caused significant reduction in OiX levels (a - $p<0.02$| b - $p<0.01$) when compared to the animals treated with saline followed by LHRH (1µg) at 24h. LH (40µg), FSH (40µg), $PGF_{2\alpha}$ (10µg), NE (1µg) or cAMP (20µg) were injected in animals injected with 10µg of LHRH 24h previously. In all these groups the levels of ODC were statistically similar when compared with respective controls treated with LH, FSH, $PGF_{2\alpha}$, NE or cAMP alone. All animals were killed at 2h after the 2nd injection. S - Represents injection of 10µl of saline. Results are mean +_ S.E.M. of 3-5 determinations from 6-10 animals.


ODC ACTIVITY (p moles/h/mg protein)
1600
1200
800
400
1 mg
10 mg
1st inj
LHRH S LHRH LHRH LHRH S LHRH S LHRH S LHRH S LHRH S LHRH
2nd inj
S LHRH LHRH LHRH LH LH FSH FSH PGF2α PGF2α NE NE CAMPCAMP

CHAPTER VI

DESENSITIZATION OF RAT TESTICULAR ORNITHINE DECARBOXYLASE TO LUTEINIZING HORMONE AND FOLLICLE STIMULATING HORMONE.

DESENSITIZATION OF RAT TESTICULAR ORNITHINE DECARBOXYLASE TO LUTEINIZING HORMONE AND FOLLICLE STIMULATING HORMONE

INTRODUCTION:

Several tissues have been shown to undergo a decrease in hormonal responsiveness following continued exposure to homologous hormone (Catt et al, 1979a; Tell et al, 1978). Prolonged exposure of Leydig cells to LH in vitro (Tsuruhara et al, 1977) induced refractoriness to a subsequent challenge with fresh hormone in terms of cAMP formation, steroid production and the number of LH/hCG receptors. In the intact ovaries or isolated follicles, prolonged exposure to LH or PGE in vitro induced refractoriness to subsequent challenge with fresh hormone and this desensitization was shown to be homospecific and dose and time dependent (Zor et al, 1976| Lamprecht et al, 1977; Lamprecht et al, 1979; Selstam et al, 1976; Ahren et al, 1980). FSH was also shown to exhibit a similar effect (Zor et al, 1976; Selstam et al, 1976; Ahren et al, 1980). Exposure of Sertoli cells to FSH in vitro causes refractoriness. During this period addition of fresh FSH does not stimulate cAMP (Verhoeven et al, 1980). FSH is alsoknown to cause partial desensitization of the FSH responsive adenyl cyclase in adult rat testis. The process is associated with a comparable decrease in specific FSH binding (Jahnsen et al, 1980).

In this chapter work on the effect of LH and FSH on desensitization of testicular ornithine decarboxylase and the effect of heterologous hormones on LH desensitized testis is presented. In addition, data on possible role of prostaglandins and catecholamines in gonadotropin stimulated ornithine decarboxylase is given.

MATERIALS AND METHODS:

The source of chemicals and animals is described in Chapter II. Some studies involving FSH were performed using ovine follicle stimulating hormone (NIH-FSH-S-12) while FSH desensitization work was done using highly purified ovine FSH (100 x NIH - FSH - S1) obtained from Dr. M.R. Sairam (sFSH). NIH-LH-S20 was used for all studies involving LH effect. Indomethacin and phenoxybenzamine were dissolved in saline:ethanol mixture (9:1) and injected intratesticularly in a volume of 5-10µl. Aspirin (200mg/kg) was given intraperitoneally in a volume of 0.1ml in propyleneglycol. All inhibitors were given 15 minutes before the injection of the hormones. Control animals received corresponding amount of the vehicle. At an appropriate time rats were killed by spinal dislocation. The decapsulated testes were homogenized in 4 volumes of homogenizing buffer and centrifuged at 25,000 x g for 30 min. The methods for the determination of protein content, [^{3}H]-leucine incorporation and ornithine decarboxylase activity are described in Chapter II.

RESULTS:

Table 1 shows stimulation of ODC in the testis in response to sFSH. Dose as low as 0.05μg caused significant stimulation of ODC at 4h. Maximum effect was observed at a dose of 0.5μg per testis.

The effect of 0.25μg and 0.5μg of sFSH on desensitization of testicular ODC is given in table 2. The results show that both doses of sFSH caused desensitization of the testis at 24h. However, desensitization of ODC activity was not observed with 40μg of NIH-S-12-FSH. This indicates that highly potent FSH is necessary to cause desensitization of the testis.

Fig. 1 shows the effect of 40μg of LH(NIH-S.20) on desensitization of ODC activity at 24h. The results show that first injection of LH caused desensitization of the testis as second injection of stimulatory dose of 40μg of LH for 4h caused significantly less stimulation of ODC activity when compared to LH alone treated animals. It was also interesting to note that injection of PGE_2 or LHRH to LH desensitized testis also showed less stimulation when compared to PGE_2 or LHRH alone treated animals. However, in the LH desensitized testis cAMP and norepinephrine caused stimulation of ODC and the values were comparable to the animals treated with cAMP and norepinephrine alone respectively. The data presented in Fig. 2

supports the view that LH causes desensitization of Leydig cells only. Injection of LH to LH desensitized testis caused significantly less ODC activity in the Leydig cell fraction while the levels of ODC in the seminiferous tubule fraction were unaltered.

The effect of indomethacin, a potent inhibitor of prostaglandin synthetase, on LH, FSH or cAMP stimulated ODC activity is given in table 3. Prior injection of indomethacin caused drastic inhibition of OX activity in FSH, LH or cAMP treated groups. To assess the possibility that indomethacin was acting at the level of general protein synthesis an experiment was conducted using H^3 leucine incorporation into testicular proteins. It was observed that FSH induced leucine incorporation was drastically inhibited by 30 and 60µg of indomethacin (table 4). The effect of aspirin, another potent inhibitor of prostaglandin synthetase at a dose of 200mg/kg body weight was also investigated. This drug did not cause inhibition of ODC activity in FSH, LH, LHRH or cAMP treated groups (table 5).

The action of phenoxybenzamine, an α-adrenergic receptor blocking agent on LH, FSH, $PGF_{2\alpha}$ and LHRH stimulated ODC activity is shown in Fig. 3. Phenoxybenzamine at a dose of 10µg/testis caused inhibition of LH and FSH stimulated OX activity. However, it did not inhibit $PGF_{2\alpha}$ and LHRH stimulated OX activity.

DISCUSSION:

The present experiments demonstrated that both FSH and LH cause desensitization of testis and that ODC is a good parameter to study this phenomenon. Testicular desensitization to gonadotropic hormones was observed earlier using cAMP and testosterone as ' parameters (Tsuruhara et al, 1977| Hsueh et al, 1977; Sharpe, 1976; Haour and Saez, 1977$ Chen and Payne, 1977)•

In this study it was observed that LH desensitized testis responds to cAMP and norepinephrine and causes stimulation of ODC comparable to the levels seen with cAMP or norepinephrine alone treated animals. It is possible that injection of cAMP to LH desensitized testis causes stimulation of ODC activity in both Leydig cells and seminiferous tubules as LH desensitization might have not disturbed the post cAMP pathway leading to the stimulation of ODC. Similarly it is possible that the pathway leading to the stimulation of ODC by norepinephrine is also not disturbed following LH desensitization in both Leydig cells and seminiferous tubules of the testis.

On the contrary it was found that injection of PGE_2 or LHRH to LH desensitized testis caused less stimulation of ODC activity when compared to PGE_2 or LHRH alone treated animals. In the previous chapters it was shown that LHRH

causes stimulation of ODC activity in Leydig cells alone while PGE_2 causes stimulation of the enzyme in both Leydig cells and seminiferous tubules. Significantly less stimulation of ODC activity by PGE_2 in LH desensitized testis could be due to the non-stimulation of Leydig cells by PGE_2 in the LH desensitized testis. This also indicates that LH desensitization of testis disturbs the stimulatory activity of PGE_2 in the Leydig cells. Since LHRH acts on Leydig cells alone, significantly less stimulation of OX observed by LHRH in the LH desensitized testis when compared to LHRH alone injected animals indicates that LH desensitlzation disturbs LHRH action on these cells. These results show that the mechanism of action of PGE_2, LH and LHRH in causing stimulation of ODC activity are probably similar. The mechanism of gonadotropic hormone desensitization has been adduced to be due to the decrease in the number of membrane receptors (Hsueh et al, 1976; Sharpe, 1976; Haour and Saez, 1977; Chen and Payne, 1977} Purvis et al, 1977), or modification of the coupling system between the receptor and the adenylate cyclase (Saez *et* al, 1978a) or blockage of enzymes beyond cAMP formation (Haour and Saez, 1978| Tsuruhara et al, 1977| Saez et al, 1978b). Desensitization of ODC activity observed in this study could be due to any one or more of these three mechanisms.

The intermediary role of prostaglandins in the action

of LH or FSH is not clear. Treatment of indomethacin, a prostaglandin synthetase inhibitor, to LH or FSH injected animals caused inhibition of ODC activity. However, similar inhibition of ODC activity by indomethacin was observed in cAMP treated animals. Indoraethacin appears to cause toxic effect on the testis since it inhibited incorporation of ^{3}H-leucine into total proteins, in addition to causing inhibition of ODC activity. Osterman et al, (1979) have also found that indomethacin caused inhibition of ODC activity and protein synthesis in the granulosa cells of ovary in vitro. Another prostaglandin synthetase inhibitor, aspirin was also tried to test the role of prostaglandins in LH or FSH action. Aspirin at a dose of 200mg/kg body weight did not inhibit LH, FSH, cAMP and LHRH stimulated ODC activity. These results indicate that ODC stimulation by the above compounds is probably not mediated by prostaglandins.

The possible involvement of catecholamines as mediators of LH or FSH action was investigated by using phenoxybenzamine, an α-adrenergic receptor blocker. Pre-injection of phenoxybenzamine caused inhibition of both LH and FSH stimulated ODC activity while it had no effect on LHRH and $PGF_{2\alpha}$ induced ODC activity. This implicates catecholamines in the mediation of FSH and LH action in the testis. Indirect evidence for the involvement of

catecholamines in the process of ovulation has been reported for a number of species (Continho and Maiz, 1971, Virutamasen et al, 1973; O'shea and Phillips, 1974; Rocereto *et* al, 1969). More recently Moudgal and Razdan (1980) have shown that incubation of hen ovarian follicles with LH in the presence of phenoxybonzamine caused inhibition of LH induced ovulation. They suggested that probably LH interacts with epinephrine or norepinephrine at the target site to induce ovulation and inhibition of this interaction of LH with catecholamines by phenoxybenzaraine causes inhibition of ovulation. The inhibitory effect of phenoxybenzamine on LH and FSH induced ODC activity in the testis is probably similar to the inhibition of ovulation in domestic hen. These results show that prostaglandins, LHRH and catecholamines, in addition to gonadotropic hormones play an important role in the regulation of testis function.

TABLE I

EFFECT OF VARIOUS DOSES OF sFSH ON ORNITHINE DECARBOXYLASE ACTIVITY ON IMMATURE RAT TESTIS

Various doses of sFSH were injected intratesticularly in 5-10µl of saline. The enzyme activity was determined at 4h as described in Materials and Methods. The values represent mean ± S.E.M. of 3-5 determinations from 6-10 animals.

Group	Dose of FSH (µg/testis)	ODC activity (pmoles/h/mg protein)
1.	Saline	609 ± 9
2.	0.05	1069 ± 99
3.	0.2	1367 ± 64
4.	0.5	1770 ± 91
5.	1.0	1599 ± 81

All treated values are significantly ($p<0.001$) different from controls. sFSH-Sairam FSH (100 x NIH-FSH-S1).

TABLE II

EFFECT OF FSH ON DESENSITIZATION OF TESTICULAR ORNITHINE DECARBOXYLASE ACTIVITY

In the first injection 0.25µg or 0.5µg of sFSH or 40µg of NIH-FSH or saline were given intratesticularly. At 24h thereafter 0.5µg of sFSH or 40µg of NIH-FSH or saline were given intratesticularly for 4h. Results are mean ± S.E.M. of 3-5 determinations from 6-10 animals.

	Treatment		ODC activity (pmoles/h/mg protein)
	1st injection	2nd injection	
1.	sFSH (0.5µg)	Saline	637 ± 115
2*	Saline	sFSH (0.5µg)	1770 ± 91
3.	sFSH (0.25µg)	sFSH (0.5µg)	1043 ± 19 [a]
4.	sFSH (0.5µg)	sFSH (0.5µg)	1054 ± 74 [a]
5.	Saline	FSH (NIH) (40µg)	1666 ± 60
6.	FSH (NIH) (40µg)	FSH (NIH) (40µg)	1636 ± 48

a-$p<0.001$ compared to group 2.

sFSH-Sairam FSH (100 x NIH-FSH-S1)

TABLE III

EFFECT OF INDOMETHACIN ON FSH, LH AND cAMP STIMULATED ODC ACTIVITY

Hormones and cAMP were injected intratesticularly in 10µl of saline. Indomethacin was injected in 5µl of ethanol. Animals in groups 1-7 were killed at 4h and in groups 8 and 9 were killed at 2h after the injection. Results are mean ± S.E.M. of 3-5 determinations from 6-10 animals per group.

Treatment	ODC activity (pmoles/h/mg protein)	Inhibition (percent)
1. Control	597 ± 14	
2. IM (60µg)	538 ± 5	
3. FSH (40µg)	1456 ± 182	
4. FSH (40µg) ± IM (30µg)	1214 ± 180	28
5. FSH (40µg) + IM (60µg)	549 ± 70	
6. LH (40µg)	1224 ± 134	100
7. LH (40µg) + IM (60µg)	609 ± 88	
8. cAMP (40µg)	879 ± 17	98
9. cAMP (40µg) + IM (60µg)	537 ± 93	
		100

Inhibition of 28% observed in group 4 is not statistically significant when compared to group 3 ($p > 0.4$).

TABLE IV

EFFECT OF INDOMETHACIN ON ^{3}H-LEUCINE INCORPORATION INTO PROTEINS OF TESTIS TREATED WITH FSH

Incorporation of ^{3}H-leucine into TCA precipitable proteins was determined at 4h after the injection of FSH and ^{3}H-leucine (5μCi/testis). The number of animals used in each group is given in parentheses. The values are mean ± S.E.M.

	Treatment		^{3}H-Leucine incorporated (cpm x 10^{2}/mg protein)
1.	Saline	(6)	669 ± 89
2.	IM (60μg)	(4)	548 ± 76
3.	FSH (40μg)	(4)	1152 ± 67
4,	FSH (40μg) + IM (30ug)	(4)	465 ± 22
5.	FSH (40μg) + IM (60μg)	(4)	396±30

TABLE V

EFFECT OF ASPIRIN ON FSH, LH, cAMP AND LHRH STIMULATED ODC ACTIVITY

Hormones and cAMP were injected intratesticularly in 10µl of saline. Aspirin at a dose of 200mg/kg body wt was injected intraperitoneally in 100µl of propane-diol fifteen minutes before the injection of the hormones. Animals were killed at 2h after the injection of hormones and cAMP. Results are mean ± S.E.M. of 3-5 determinations from 6-10 animals per group.

	Treatment	ODC activity (pmoles/h/mg protein)
1.	Control	709 ± 64
2.	Aspirin	835 ± 42
3.	FSH (40µg)	1214 ± 113
4.	FSH (40µg) + Aspirin	1125 ± 40
5.	LH (40µg)	1224 ±134
6.	LH (40µg) + Aspirin	1127 ± 71
7.	LHRH (1µg)	1260 ± 85
8.	LHRH (1µg) + Aspirin	1547 ± 160
9.	CAMP (20µg)	1312 ± 58
10.	cAMP (20ug) + Aspirin	1382 ± 52

None of the aspirin treated groups were statistically different from the hormone or cAMP treated groups.

Fig. 1 Effect of LH on desensitization of testicular ODC. LH (40μg/testis) was injected intratesticularly and 24h after this injection a second injection of LH (40μg/testis) was given and the animals were killed 4h later for the estimation of ODC activity. Prior treatment with LH caused reduction in ODC activity when compared with the animals treated with LH alone (a - $p<0.001$). PGE_2 (10μg/testis), cAMP 20μg/testis), norepinephrine (1μg/testis) and LHRH (1μg/testis) were treated to the animals which were exposed to LH (40μg/testis) 24h previously. These animals were killed 2h after the second injection for the estimation of ODC activity. S - Indicates injection of 10μg of saline. b and c - Significantly different from their corresponding controls ($p<0.01$). Results are mean ± S.E.M. of 3-5 determinations from 6-10 animals per group.

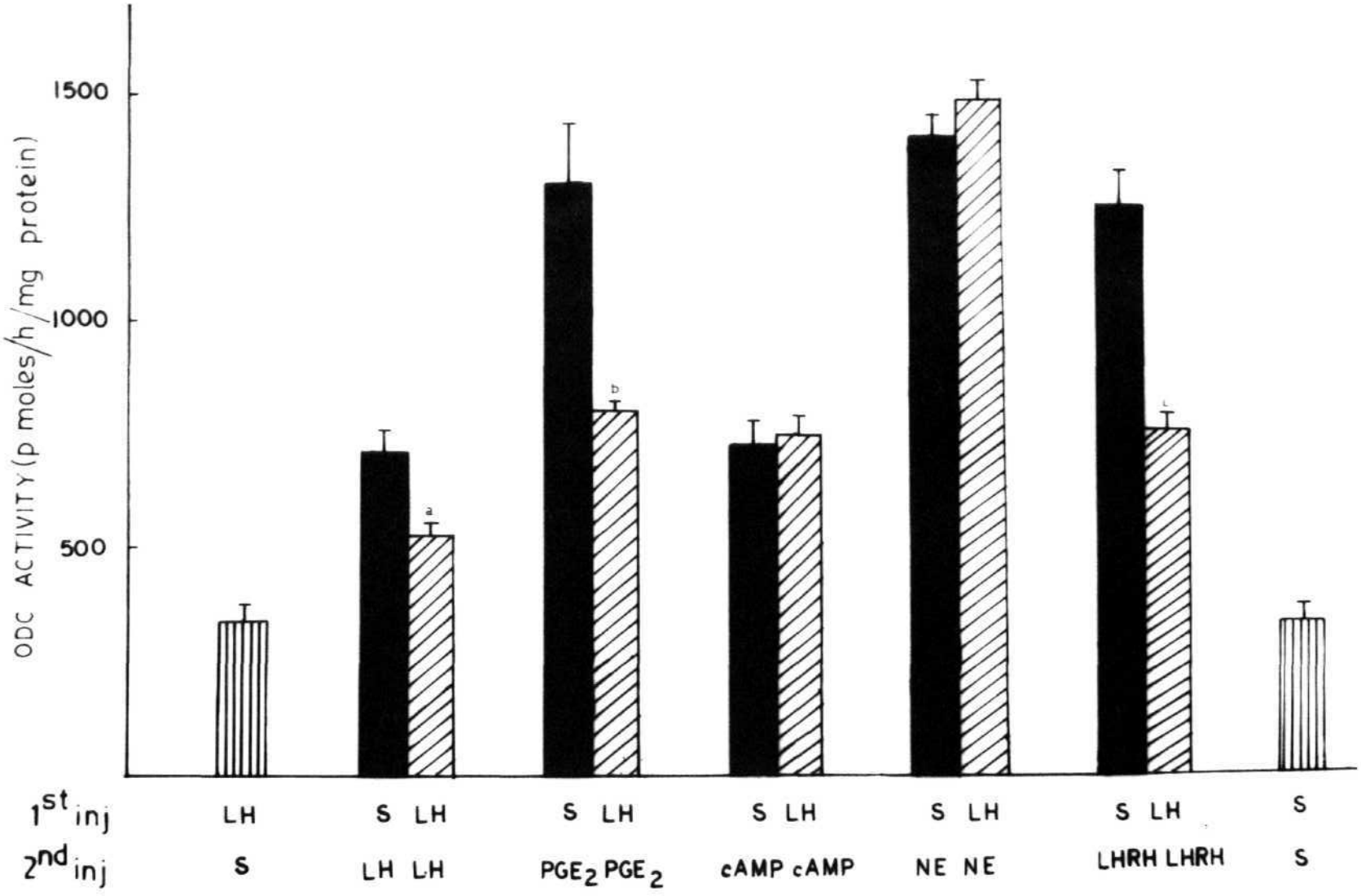

ODC ACTIVITY (p moles/h/mg protein)
1500
1000
500
a
b
c
1st inj
2nd inj
LH
S
S LH
LH LH
S LH
PGE2 PGE2
S LH
cAMP cAMP
S LH
NE NE
S LH
LHRH LHRH
S
S

Fig. 2 Effect of LH on desensitization response in Leydig cell and seminiferous tubule fractions. The animals were injected intratesticularly with 40μg of LH/testis. 24h later a second injection of LH (40μg/testis) was given and the animals were killed 4h later for the assay of ODC in Leydig cells and seminiferous tubules as described in materials and methods. a - Significantly different from LH alone injected Leydig cell fraction ($p<0.001$). Results are mean ± S.E.M. of 3-5 determinations from 18-20 animals per group. S - Represents injection of 10μl of saline.

no

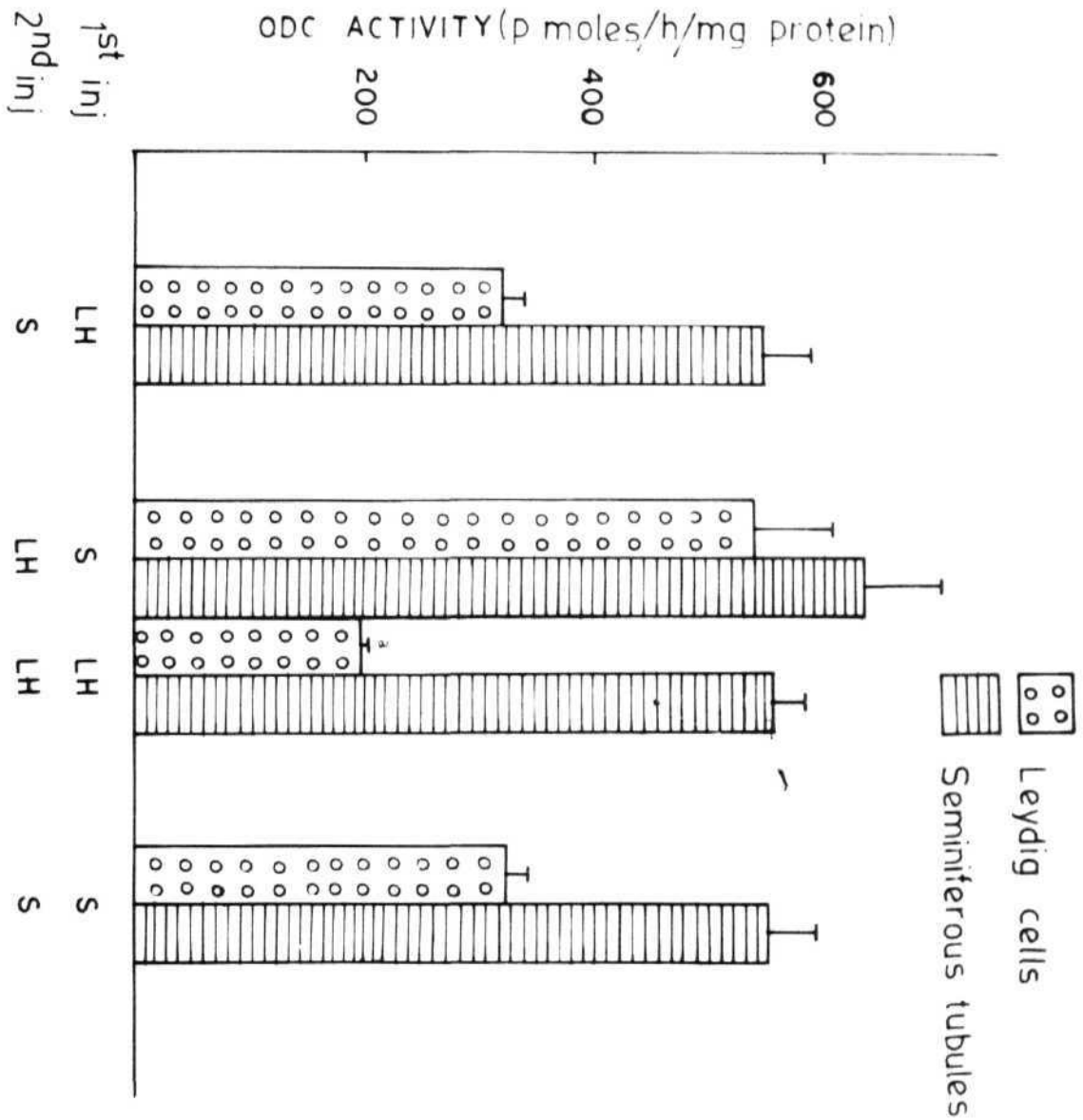
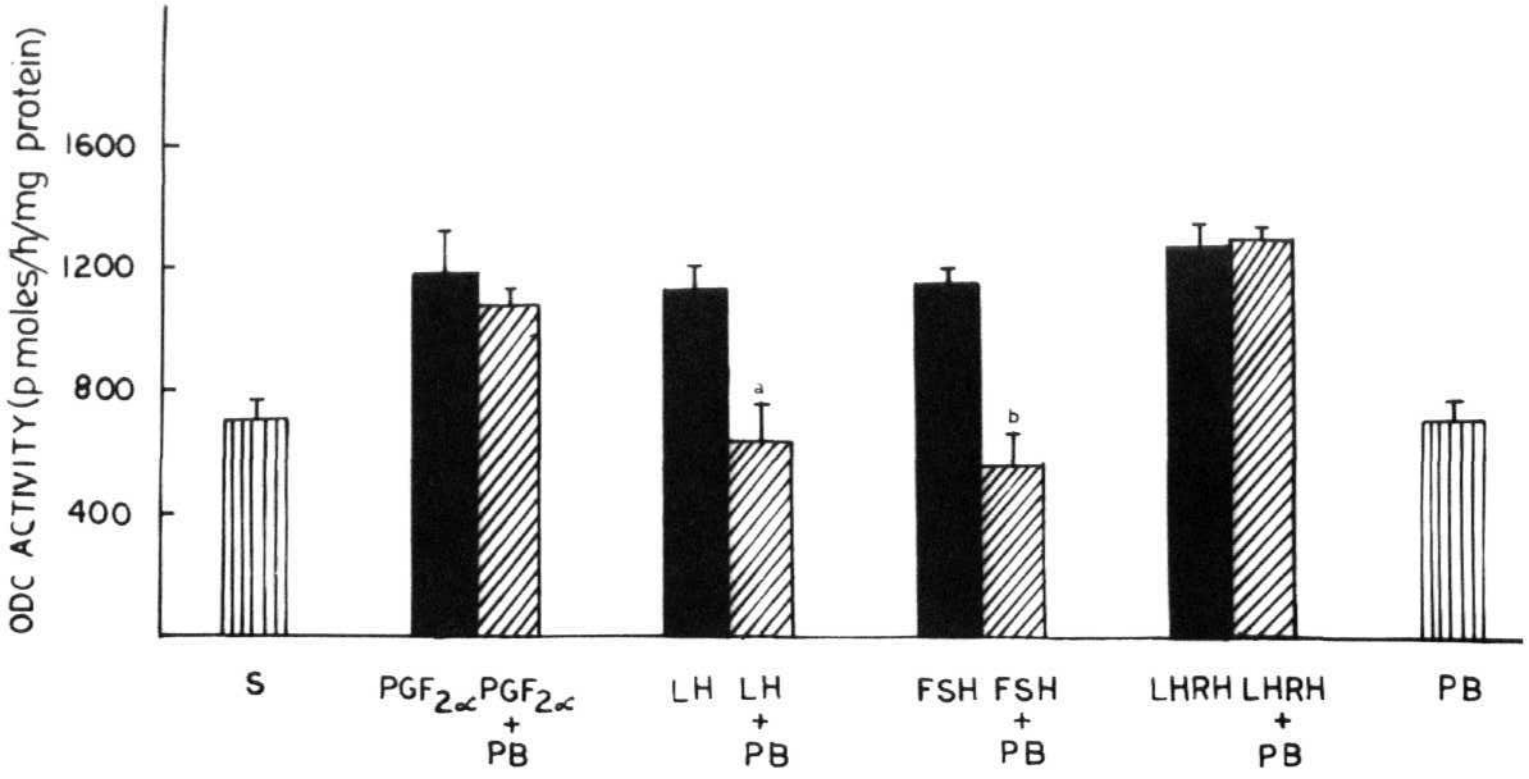

ODC ACTIVITY (p moles/h/mg protein)
200
400
600
1st inj
2nd inj
S
LH
S
LH
LH
LH
S
S
Leydig cells
Seminiferous tubules

Fig. 3 Effect of phenoxybenzamine on LH, FSH, $PGF_{2\alpha}$ and LHRH stimulated ODC activity. Phenoxybenzamine (10µg/testis) was injected intratesticularly 15 min before the injection of the above compounds in 5-10µl of saline-ethanol (9:1, v/v) mixture. LH (40µg/testis) or LHRH (1.0µg/testis) were also injected intratesticularly and the animals killed 2h later for the estimation of ODC. Results are mean ± S.E.M. of 3-5 determinations consisting of 6-10 animals per group. a and b - Significantly different when compared with corresponding controls ($p<0.001$). S - Represents saline (10µl) treated controls.


ODC ACTIVITY (p moles/h/mg protein)
1600
1200
800
400
a
b
S
PGF2α
PGF2α + PB
LH
LH + PB
FSH
FSH + PB
LHRH
LHRH + PB
PB

CHAPTER VII

EFFECT OF α-DIFLUOROMETHYLORNITHINE ON THE TESTIS.

EFFECT OF tt-DIF LUOROM ETHYLO RN ITHIN E ON THE TESTIS

INTRODUCTION:

Ornithine decarboxylase catalyses the first and rate limiting step in the biosynthesis of the polyamines putrescine, spermidine and spermine in animal tissue (Pegg et al, 1970* Russell, 1973). Since polyamines have been implicated in the regulation of cell growth (Russell, 1973| Mamont et al* 1976), attempts have been made to inhibit ODC in order to clarify its role. The current interest in elucidating the intracellular roles of the polyamines had led to the synthesis and investigation of a number of inhibitors of ODC. Metcalf et al. (1978) showed that DL-α-difluoromethylornithine first synthesized by Bey and Vevert (1978), is an enzyme-activated irreversible inhibitor of mammalian 5DC. In rat DFMO reduced ODC activity in a dose-dependent and time dependent manner in ventral prostate, testis and thymus (Danzin et al, 1979a). Administration of DFMO to mice and rats during early gestation suppressed the levels of ODC and arrested embryonic development (Fozard et al, 1980a; Reddy and Rukmini, 1981). Recent studies have indicated that the rise in ODC activity may be related to subsequent DNA synthesis and cell proliferation in various tissues in vivo (Inoue et al, 1975 ; Ka to *et* al, 1976; Takigawa et al, 1977; Poso and Janne, 1976), and in cultured cell systems in vitro. (Relyea and

Rando, 1975; Mamont et al, 1976). Mamont et al (1978) reported that DFMO treatment results in inhibition of proliferation of rat hepatoma, L1210 murine leukemia, and human prostatic adenoma in vitro*

Studies reported in this chapter were undertaken with an aim to see if DFMO causes inhibition of testis function through inhibition of ODC levels.

MATERIALS AND METHODS:

The source of the chemicals and animals are given in Chapter II. In the first set of experiments immature male rats (25-30g) aged 21-22 days were used. DFMO (400mg/kg) in 0.2ml of saline was injected intraperitoneally 15 minutes before the injection of PGE_2. PGE_2 (10µg/testis) was given intratesticularly using an Unimatrix syringe. Saline treated rats served as controls. All animals were killed by cervical dislocation and ODC activity of the testes was measured at required time interval as described in Chapter II.

In a second set of experiments 20 male rats aged 15 days were divided into two batches. Batch I consisting of 10 males were treated with DFMO (5mg/rat) intraperitoneally in a volume of 0.1ml of saline twice daily for 10 days. Batch II served as control and received two daily injections of saline. Batch I animals were given an injection of DFMO

(10mg/rat) 2h before sacrifice on day 25 of age and simultaneously 5μCi of ^{3}H-thymidine or ^{3}H-leucine was given intxatesticularly. Control animals also received the precursor similarly 2h before killing. Testes were removed, weighed and used for the extraction and estimation of DNA, RNA and protein and for the incorporation of radioactive precursors as mentioned in Chapter II.

A similar experiment was performed using 35 day old animals. The dose of DFMO for these animals was maintained at 200mg/kg body wt.

RESULTS:

Fig. 1 shows the effect of a single dose of DFMO (400mg/kg body weight) on PGE_2 stimulated ODC activity at various time intervals. At 4h after the injection of DFMO there was a significant fall in PGE_2 stimulated ODC activity. Maximum inhibition of PGE_2 stimulated OX occurred at 12h after DFMO treatment. Thereafter, even at 48h, the enzyme activity did not recover to its normal value.

Fig. 2 shows that the inhibition of ODC following treatment with DFMO was dose-dependent. A dose as low as 20mg/kg body weight caused a significant inhibition of PGE_2 stimulated ODC activity.

Daily intraperitoneal treatment with DFMO from day 15 after birth until day 25 had no influence on net body weight and the weight of the testis (table 1). DFMO did not have any effect on the incorporation of [^{3}H]-thymidine into DNA and that of [^{3}H]-leucine into proteins in the above treated animals. Further, there was no significant difference between control and treated animals with respect to DNA, RNA and protein content (table 1).

Similarly when DFMO (200mg/kg body weight) was given intraperitoneally in rats from day 35 after birth until day 45 no effect was observed on the testicular weight, RNA, DNA, protein and on the incorporation of the two precursors (table 2).

DISCUSSION:

The work presented in this chapter shows that DFMO, an enzyme activated irreversible inhibitor of ODC, inhibits PGE_2 stimulated ODC *in vivo*. The inhibition by DFMO is both dose and time dependent. Earlier work by Danzin *et al* (1979) showed that DFMO inhibits ODC activity in testis. However, in their experiments ODC activity increased slowly at 24h after the injection of DFMO.

Fozard *et al* (1980b) observed that in mice treatment with DFMO during days 5-8 of pregnancy caused depletion of

putrescine and spermidine levels in the embryonic regions of the uterus. This depletion of polyaminies was paralleled by the depletion of RNA, DNA and protein content in the embryonic regions. Similar results on the reduced levels of ODC were also observed by Reddy and Rukmini (1981) and these authors have suggested that since embryonic resorption occurs due to depletion of polyamine levels, DFMO may act as a postcoitally effective antifertility agent. In the present study testicular nucleic acid levels and protein content were not altered following treatment with DFMO. This indicates that the depletion of OX levels may not cause drastic effects on the testis of rat.

TABLE I

EFFECT OF TREATMENT WITH DFMO ON THE TESTIS OF 15 DAY OLD RATS

	CONTROL Mean ± SEM	EXPERIMENTAL Mean ± SEM
Body wt. (g)	23.7 ± 1.64(10)	22.5 ± 1.40(10)
Testis wt. (mg)	66 ± 12.03(10)	64.15± 14.37(10)
Total DNA (µg/testis)	270.90± 15.16(6)	279.59± 13.39(6)
Total RNA (µg/testis)	431.77± 43.29(6)	377.83± 56.87(6)
Total protein (mg/testis)	2.42± 0.21(6)	2.75± 0.27(6)
^{3}H-Thymidine incorporation (cpm/mg testis)	1549 ±329 (4)	1364 ±129 (4)
^{3}H-Leucine incorporation (cpm/mg testis)	1714 ±227 (4)	1411 ±240 (4)

DFMO was given intraperitonially for 10 days at a dose of 5mg/rat, twice a day. Control animals received saline. 2h before killing one testis was injected with 5µCi of ^{3}H-thymidine and another testis was injected with 5µCi of ^{3}H-leucine. Incorporation of the precursors into TCA precipitable material was measured as described. The number in parenthesis indicates the number of animals.

TABLE II

EFFECT OF TREATMENT WITH DFMO ON THE TESTIS OF 35 DAY OLD RATS

	CONTROL Mean ± SEM	EXPERIMENTAL Mean ± SEM
Body wt.(g)	46.81± 2.6(10)	48.54± 4.2(10)
Testis wt. (mg)	177.61± 28.8(10)	152 ± 26.86(10)
Total DNA (µg/testis)	474.56± 31.34(6)	614.27± 51.64(6)
Total RNA (µg/testis)	650.83± 67.38(6)	681.69± 58.50(6)
Total protein (mg/testis)	4.41± 0.32(6)	3.49± 0.25(6)
^{3}H-Thymidine incorporation (cpm/mg testis)	174 ± 64 (4)	194 ± 61 (4)
^{3}H-Leucine incorporation (cpm/mg testis)	1983 ±658 (4)	2315 ±846 (4)

DFMO was injected intraperitonially for 10 days at a dose of 200 mg/kg, twice a day. Control animals received saline. 2h before killing one testis was injected with 5µCi of ^{3}H-thymidine and another testis was injected with 5µCi of ^{3}H-leucine. Incorporation of the precursors into TCA precipitable material was measured as described. The number in parenthesis indicates the number of animals.

Fig. 1 Effect of DFMO on PGE_2 stimulated OX activity. DFMO (400mg/kg body wt) was injected intraperitoneally at 4, 12, 24 or 48h before intratesticular injection of 10µg of PGE_2. Animals were killed at 2h after PGE_2 injection for the assay of ODC activity. Results are mean ± S.E.M. of 3-5 determinations from 6-10 animals per group. Arrow represents ODC value of DFMO (400mg/kg body wt) treated animals at 2h.

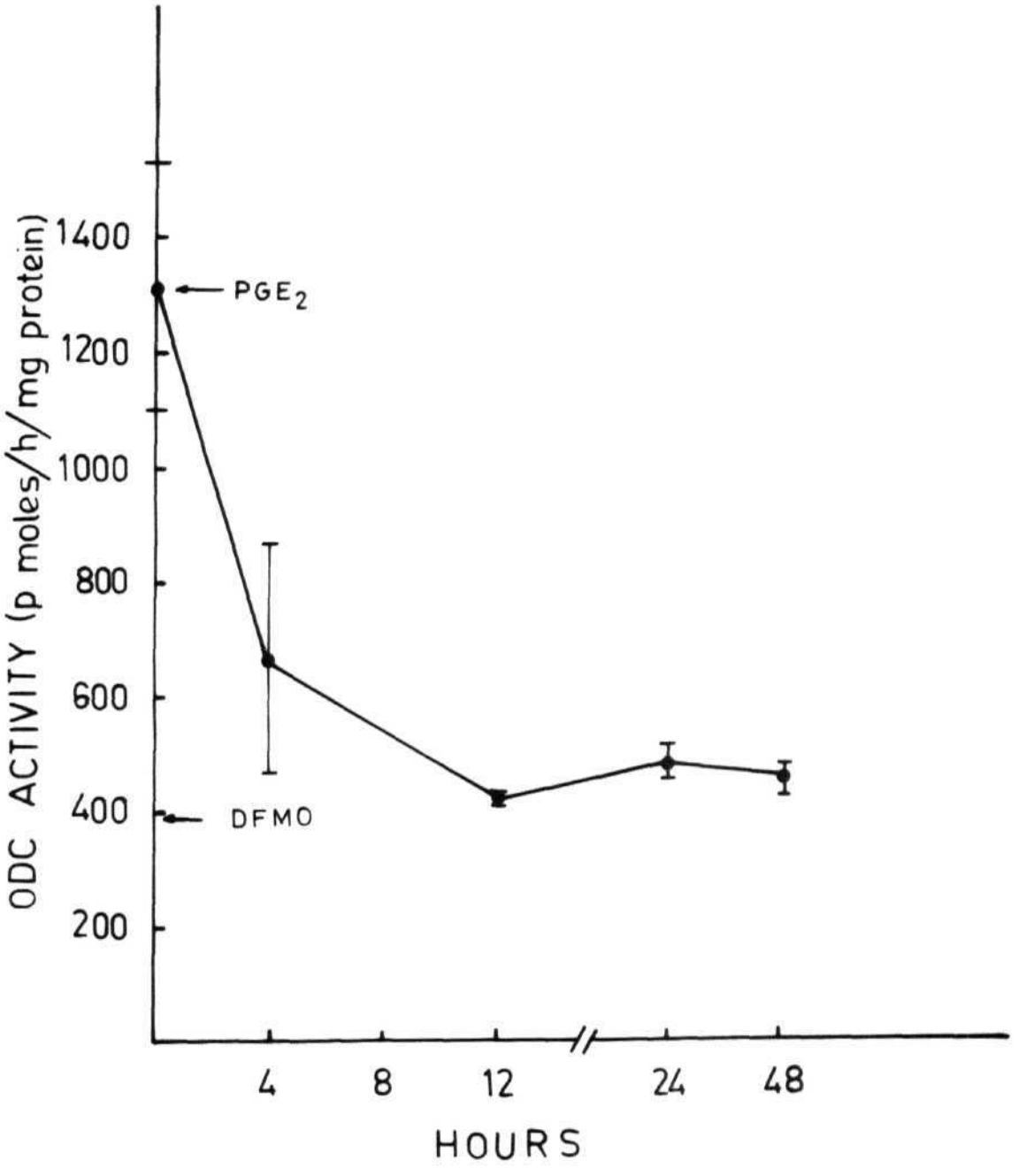
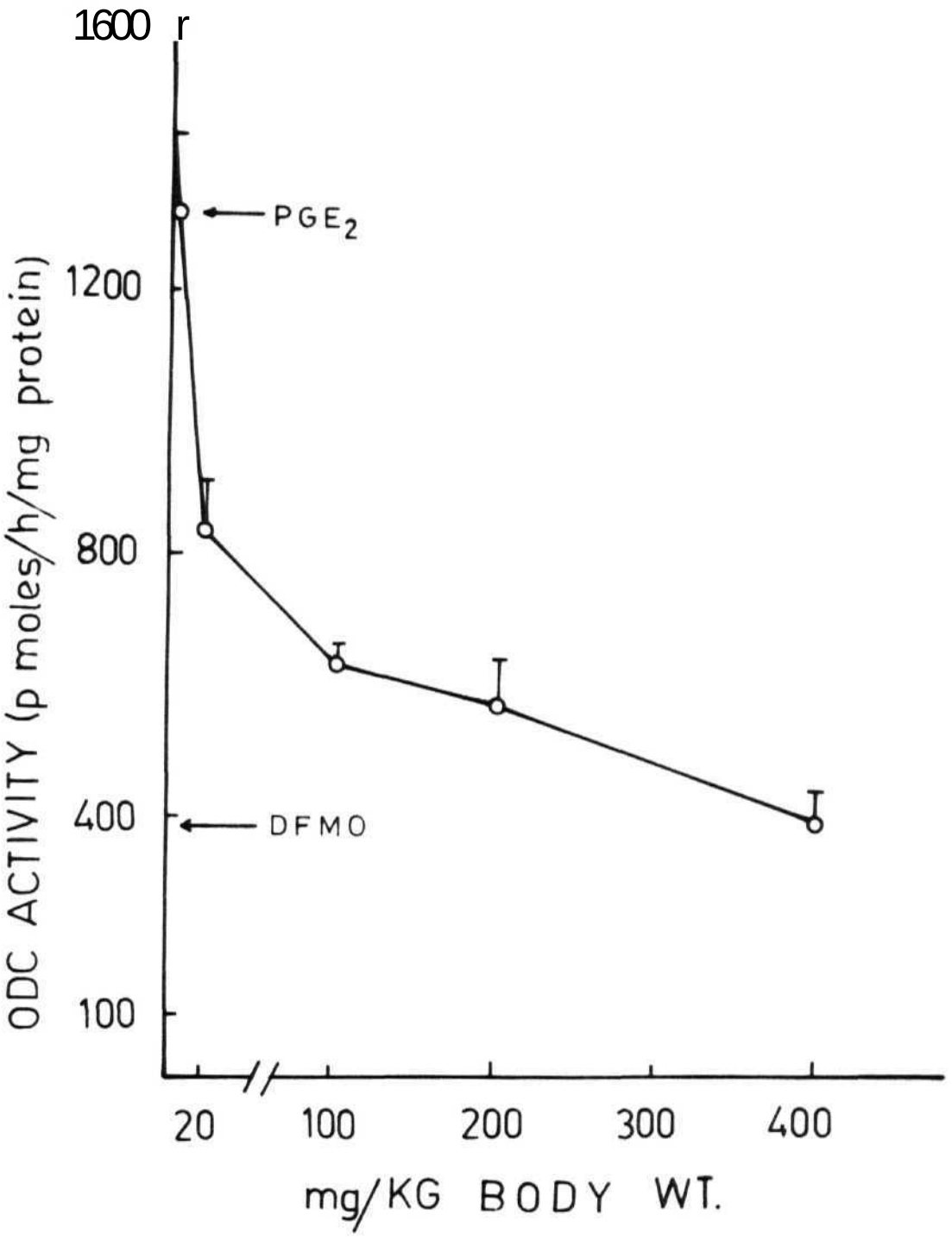

ODC ACTIVITY (p moles/h/mg protein)
1400
1200
1000
800
600
400
200
PGE2
DFMO
4
8
12
24
48
HOURS

Fig. 2 Effect of different doses of DFMO on PGE_2 stimulated ODC activity. Rats were injected intraperitoneally with different doses of DFMO, 15 min before the intratesticular injection of PGE_2 (1Oμg/testis). Following this the animals were killed at 2h for the assay of ODC activity in the testis. Results are mean ± S.E.M. of 3-5 determinations from 6-10 animals per group. Arrow represents DFMO (400mg/kg) alone treated group at 2h.


1600
1200
800
400
100
PGE2
DFMO
ODC ACTIVITY (p moles/h/mg protein)
20
100
200
300
400
mg/KG BODY WT.

GENERAL DISCUSSION

GENERAL DISCUSSION

The endocrine and reproductive functions of the gonads are maintained and regulated by the actions of follicle-stimulating hormone and lutenizing hormone. In higher vertebrates, FSH and LH exert characteristic effects upon specific target cells in the testis and ovary. The actions of LH are predominantly directed for steroidogenesis and ovulation, while FSH acts upon germ cells causing their development and maturation. It is now well recognized that many, if not all, of the effects of gonadotropic hormones are mediated by cAMP. LH and FSH have been shown to increase cAMP levels in the Graafian follicles (Tsafriri *et al*, 1972a), granulosa cells (Kolena and Channing, 1972) and corpora lutea (Marsh *et al*, 1966) of the ovary and in the Leydig cells and Sertoli cells (Means et al, 1974; Dorrington et al, 1974b) of the testis.

Besides gonadotropic hormones, prostaglandins and catecholamines also appear to be involved in ovarian function. Exogenous prostaglandins mimic most of the biological and biochemical responses of LH in ovary in vivo and in vitro (Speroff and Ramwell, 1970; Marsh, 1971; Osterman and Hammond, 1978). The present study shows that prostaglandins and catecholamines play an important role in the modulation of ODC in the testis. Kuehl et al (1970b)

advanced the hypothesis that PGE is interposed between LH and cAMP in the regulation of steroidogenesis and thereby functions as a second messenger instead of cAMP. However, various other experimental data argue against this theory. The stimulatory action of LH on cAMP is a rapid one (few minutes), while the effect of LH on PGE production was observed only after 2-5h (LeMaire et al, 1973| Marsh et al, 1974; Bauminger and Lindner, 1975). The precise position of PGE in the regulation of steroidogenesis still remains to be established. In the present study prostaglandins were found to cause stimulation of cAMP and OX activity in the testis. Stimulation of cAMP was observed at 30 rain after the injection of the prostaglandins while ODC levels were found to increase at a much later time of 2h. In addition to this, MIX a phosphodiesterase inhibitor, caused an additive effect with PGE_2. These observations indicate that the action of prostaglandins may be mediated by cAMP in the testis of rat,

Catecholamines also appear to play an important role in ovarian function. Both rat and rabbit corpus luteum have been found to contain a gonadotropin responsive adenyl cyclase system that is also responsive to β-adrenergic catecholarnines (Birnbaumer et al, 1976). They have observed that stimulatory effects of LH and catecholamine were not additivef hence they concluded that the same adenyl

cyclase system is responsive to both of these agents. In our study stimulation of ODC by catecholamines appears to be mediated through a mechanism involving α-adrenergic receptors. Furthermore, since α-adrenergic blocking agents inhibit FSH and LH induced ODC activity, catecholamines appear to be involved in the mediation of these gonadotropic hormones in the testis#

Another feature of the present investigation is the regulation of testicular function by luteinizing hormone releasing hormone. In the recent years it has been observed that LHRH and its agonists act directly on the testis. Most of the direct effects of LHRH observed in the testis and in ovary appear to be inhibitory. However, the effect of LHRH and its analogs on granulosa cell vary from inhibitory to stimulatory depending upon the experimental conditions used and parameters examined. In the present study, LHRH was found to increase testicular ODC levels in the Leydig cell fraction. The mechanism of action of LHRH in causing stimulation of ODC is paradoxical. Recently the stimulatory effects of LHRH in granulosa cells were shown to be associated with increased levels of prostaglandins (Clark et al, 1980; Clark, 1982). This gives rise to the possibility that LHRH stimulation of ODC activity in the testis is probably mediated by prostaglandins. This possibility

could not be confirmed as aspirin, a prostaglandin synthetase inhibitor did not inhibit LHRH induced ODC activity. Thus the mechanism of induction of ODC by LHRH needs further elucidation.

Another notable feature of the present work is the phenomenon of desensitization of testicular ODC by gonadotropic hormones, prostaglandins, catecholamines and LHRH. During desensitization the initial loss of measurable receptors appears to be due to receptor occupancy by the hormones where as the secondary refractory state is accompanied by real loss of receptors (Catt et al, 1979c). Studies on the kinetics of desensitization reaction indicate that down regulation of the receptors may not be the only factor involved in the decrease of hormone dependent adenyl cyclase activity (Johnson et al, 1978; Saez et al, 1979| Su et al, 1979). The other mechanisms involved may be modification of the coupling system between receptor and adenyl cyclase (Saez et al, 1978a) or blockage of some step beyond cAMP formation (Haour and Saez, 1978; Tsuruhara et al, 1977; Saez et al. 1978b). The synthesis of an endogenous protein inhibitor triggered by agonist may also be necessary for the development of the refractory state. Such an inhibitor appears to act by interfering with the coupling of the hormone-receptor complex to the catalytic subunit of adenyl cyclase (Lamprecht *et al*, 1979). In ttiis study, both types

of desensitization namely homologous and heterologous, have been observed. The mechanism of desensitization in this study appears to be due to inhibition of ODC at a step before cAMP formation for epinephrine and due to a post cAMP block in the case of prostaglandins.

Polyamines are mainly associated with growing and embryonic tissue. Recent studies have indicated that tht rise in ODC activity may be related to cell proliferation in various tissues in vivo (Inoue et al, 1975$ Kato et al, 1976; Takigawa et al, 1977| Poso and Janne, 1976). Testi» is an actively dividing organ. This study shows that DFMO causes reduction in the levels of ODC in the testis. However, reduced levels of ODC do not cause any effect on the content of DNA, RNA and protein, Fozard et al (personal communication) have also observed that DFMO at a dose of 200mg/kg given every six hours, for 5 days did not affect the histology of the testis. Thus depletion of polyamine levels in the testis appear to cause no effect on spermatogenesis in rats.

SUMMARY

SUMMARY

1. Intratesticular injection of prostaglandin E_2 and $F_{2\alpha}$ caused stimulation of ornithine decarboxylase activity in the testis of immature rat. This effect was both dose and time dependent.

2. Dibutyryl cyclic AMP and 1-methyl, 3-isobutyl xanthine, a phosphodiesterase inhibitor also stimulated ODC activity. Injection of PGE_2 in addition to MIX or cAMP caused increased stimulation of ODC. Stimulation of cAMP levels occurred at 30 min after the injection of PGE_2 while ODC levels were maximally increased at a much later time of 2h. These results indicate that cAMP may mediate the stimulatory action of PGE_2.

3. Simultaneous injection of gonadotropic hormones in combination with prostaglandins resulted in additional stimulation of ODC activity. This hyperstimulation may be due to the action of these compounds on different types of cells in the testis.

4. The effect of PGE_2 and $PGF_{2\alpha}$ on ODC levels in the Leydig cell and seminiferous tubule fractions were also monitored. These compounds stimulate ODC levels in both Leydig cells and seminiferous tubules.

5. Exposure of the testis to PGE_2 or $PGF_{2\alpha}$ 24h previously rendered the testis refractory to second injection with PGE_2 of $PGF_{2\alpha}$. Refractoriness to PGE_2 was dose and time dependent.

6. $PGF_{2\alpha}$ at a higher dose of 50µg/testis caused desensitization of testis in terms of cAMP formation.

7. The effect of LH, FSH and cAMP in the PGE_2 desensitized testis showed that the testis is refractory to these heterologous agents in terms of ODC response.

8. PGE_2 desensitization appears to cause the inhibitory effect at a step beyond cAMP formation.

9. In addition to prostaglandins the catecholamines epinephrine, norepinephrlne and isoproterenol also caused stimulation of ODC activity in the testis of immature rat. Like prostaglandins the effect of epinephrine was also time and dose dependent. The minimal effective dose for epinephrine was found to be 100pg and optimal stimulation was observed with 500ng of the drug per testis.

10. Simultaneous injection of epinephrine with cAMP, LH FSH or PGE_2 caused additional stimulation of the enzyme

activity. Furthermore, injection of epinephrine to norepinephrine treated animals also caused additional effect. This appears to be due to the action of these compounds on different types of cells causing hyper-stimulation. Additional stimulation of CDC activity observed in this study following treatment with norepinephrine to epinephrine treated animals may be due to the action of these two catecholamines on two different types of receptors in the same cell or due to their action on entirely different populations of the cells in the testis.

11. Both epinephrine and norepinephrine stimulate the enzyme activity in Leydig cells and seminiferous tubule fractions.

12. Studies on the effect of a and β-adrenergic receptor antagonists on epinephrine stimulated CDC activity showed that β-adrenergic receptor antagonists, propranolol and practolol did not inhibit epinephrine stimulated CDC activity while α-adrenergic receptor antagonist, phenoxybenzamine blocked epinephrine stimulated CDC activity. This indicates that CDC induction in the rat testis is mediated through a mechanism involving a-adrenergic receptors.

13. Prior exposure of the testis to norepinephrine caused desensitization of testis in terms of ODC activity. The refractoriness of the testis to norepinephrine was dose-and time dependent.

14. Norepinephrine desensitized testes were found to be fully responsive to $PGF_{2\alpha}$, LH, FSH and cAMP. On the other hand $PGF_{2\alpha}$ desensitized testes were found to be refractory to norepinephrine, from these results it appears that $PGF_{2\alpha}$ and norepinephrine act through different membrane receptors due to their structural dissimilarities. As a result the sequence of events leading to desensitization with $PGF_{2\alpha}$ and norepinephrine may be different.

15. The effect of LHRH on ODC activity in immature rat testis was also studied. The results show that LHRH acts directly on testis and stimulates ODC activity.

16. LHRH stimulated the ODC activity in a dose and time dependent manner.

17. Injection of maximal effective dose of LHRH along with $PGF_{2\alpha}$, LH and cAMP caused hyperstimulation of ODC activity.

18. LHRH caused significant stimulation of ODC activity in Leydig cell fraction only indicating that this decapeptide acts only on the Leydig cells.

19. Prolonged exposure to LHRH caused desensitization of testis in terms of ODC activity. This phenomenon was specific only to LHRH as all the other treated compounds LH, FSH, PGF_{2a}, norepinephrine and cAMP caused stimulation of GDC in LHRH desensitized testis.

20. Exposure of the testis individually to both LH or FSH caused the testis refractory to these hormones. The desensltization effect by LH was confined to Leydlg cells.

21. The testis desensitized with LH responded to CAMP and norepinephrine and caused stimulation of ODC activity. It is possible that the pathway leading to the stimulation of ODC by norepinephrine and cAMP is not distributed following LH desensitization. On the other hand it was observed that injection of PGE2 or LHRH to LH desensitized testis caused significantly reduced stimulation of ODC activity. Less stimulation of ODC activity by PGE_2 in LH desensitized testis could be due to the non-stimulation of Leydig cells by PGE_2 since LH causes desensitization of Leydig cells only. LH desensitization appears to disturb the action of LHRH on Leydig cells as injection of LHRH to LH desensitized testis did not cause stimulation of ODC activity.

22. The possible intermediary role of prostaglandins in the action of LH and FSH was studied by prior treatment with prostaglandin synthetase inhibitors, indomethacin and aspirin. Indomethacin caused inhibition of FSH induced ODC activity. However, this effect appears to be due to the toxic effect of this compound. Aspirin at a dose of 200mg/kg body weight did not inhibit LH, FSH, cAMP and LHRH stimulated ODC activity. These results indicate that ODC stimulation by these compounds is probably not mediated by prostaglandins.

23. The involvement of catecholamines in the action of LH and FSH was also studied by using catecholamine inhibitor phenoxybenzamine, an α-adrenergic receptor blocker. It caused inhibition of both LH and FSH stimulated ODC activity. However, it had no effect on LHRH and $PGF_{2\alpha}$ induced ODC activity.

24. The effect of α-diflouromethyl ornithine, a specific irreversible inhibitor of ODC activity on PGE_2 stimulated ODC activity was studied in immature rat testis. Prior injection of DFMO caused inhibition of ODC in a dose dependent manner.

25. However, treatment of DFMO did not cause reduction in the weight of the testis and the content of DNA, RNA and protein.

26. These observations show that in addition to gonadotropic hormones, catecholamines, prostaglandins and LHRH play an important role in the regulation of testis function.

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APPENDIX

The following papers were published from this thesis.

1. Effect of prostaglandins on ornithine decarboxylase activity in the testis of immature rat.
   R. Madhubala and P.R.K. Reddy
   Prostaglandins (1980) 20, 503.

2. Stimulation of ornithine decarboxylase activity by prostaglandins in the isolated cells of immature rat testis.
   R. Madhubala and P.R.K. Reddy
   FEBS Letters (1980) 122-197.

3. Effect of catecholamines on ornithine decarboxylase activity in the testis of immature rat.
   R. Madhubala and P.R.K. Reddy
   Biochem. Biophys. Res. Commun. (1981) 102, 1096.

KFI :T OF PROSTAGLANDINS ON ORNITHINE DECARBOXYLASE ACTIVITY IN THE TESTIS OF IMMATURE RAT

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ABS RACT

Intrateeticular injection of prostaglandin E_2(PGE_2) and $F_{2\alpha}$ ($PGF_{2\alpha}$) caused stimulation of ornithine decarboxylase (ODC) activity in the testis of immature rats. PGE_2 at a dose of 10 µg per testis was maximally effective 2 hours after the injection. Dibutyryl cyclic AMP (cAMP) and 1 methyl, 3-isobutyl xanthine (MIX), a phosphodiesterase inhibitor, also stimulated ODC activity. Simultaneous injection of PGE_2 and FSH or LH caused additional stimulation of ODC activity. Similarly injection of PGE_2 in addition to cAMP or MIX also caused increased stimulation of ODC. Indomethacin (IM, 60 ug/testis) inhibited LH, FSH or cAMP induced ODC activity. However, IM at the same dose inhibited the synthesis of total proteins. These results suggest that PGE_2 and $PGF_{2\alpha}$ stimulate the activity of ODC. The action of prostaglandins may be independent of the action of gonadotropic hormones. cAMP appears to mediate the action of prostaglandins in the testis of rat.

INTRODUCTION

Prostaglandins are implicated in several functions of male reproductive system. They affect the contraction of testicular capsule *(X)* and have an effect on testicular blood flow (2). Prostaglandins mimic the action of LH in stimulating the synthesis of testosterone (?) and cAMP (4) in dispersed rat interstitial cells. However, prostaglandins were also shown to inhibit LH induced testosterone synthesis in the Leydig cells of rat (4) and hCG induced steroidogenesis in the decapsulated testis of mouse in vitro (5).

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ProBtaglandins stimulate ODC activity in the ovary of rat *in vivo* (6) and in granulosa cells in vitro (7). They have been implicated to play an intermediary role in the action of gonadotropins in ovary (8). In an earlier study we have observed that gonadotropic hormones and cAMP stimulate the levels of ODC in the testis of immature rat (9). The present work shows that PGE_2 and $PGF_{2\alpha}$ mimic the action of gonadotropic hormones and cause stimulation of ODC activity in the testis of immature rats *in vivo*.

MATERIALS AND METHODS

Ovine luteinizing hormone (NIH-LH-S-20), ovine follicle stimulating hormone (NIH-FSH-S-12) and prostaglandins were obtained from the National Institutes of Health and the Upjohn company respectively. Dibutyryl cAMP, indomethacin, ornithine, pyridoxal phosphate, dithiothreitol (DTT) and other fine chemicals were purchased from Sigma Chemical Go, 1 methyl, 3-isobutylxanthine waa obtained from Aldrich Chemical Company, DL-($1^{14}C$) ornithine monochloride (58 mCi/m mol) was purchased from Radio chemical Centre, Amersham and ^3H-L-leucine (3.3 Ci/mmol) was purchased from Bhabha Atomic Research Centre, India. All other chemicals were of analytical grade and were purchased locally.

Stock solutions of prostaglandins were prepared in ethanol at a concentration of 10mg/ml. It was diluted with 9 volumes of saline before use. Indomethacin was dissolved in ethanol (60 µg/ 5 µl) and was used immediately. Stock and working solutions of hormones, cAMP and MIX were made in saline.

Immature male rats, derived from Wistar strain, aged 21-22 days, weighing between 25-30 *g* were used throughout this study. The animals were injected intratesticularly using a Unimatrix syringe with a 27 gauge needle as described earlier (9). Prostaglandins, FSH, LH, cAMP, MIX or indomethacin were injected in a total volume of 5-10 µl per testis. Control animals received 10 µl of saline, 5 µl of ethanol or both. At an appropriate time the rats were killed by spinal dislocation. The decapsulated testes were homogenised in 4 volumes of TED buffer (Tris 25 mM, EDTA 0.1 mM, DTT 1.0mM; PH 7.4) in an all glass homogenizer (Kontes) and centrifuged at 25,000 x g for 30 min. The supernatant waa used for the assay of ODC activity as described by Janne and Williams-Ashman (10). The assay was carried out in glass tubes equipped with rubber stoppers from which glass center wells containing 0.1 ml of Beckman tissue solubilizer were suspended. The incubation

mixture contained 0.5 µ moles of unlabelled ornithine, 2.5 µ moles of DTI, 0.1 µ moles of pyridoxal phosphate and 0.2 uCi of radioactive ornithine (250,000 CPM) and 200 µl of enzyme in a final volume of 0.5 ml. The tubes were incubated at 37°C for 60 min and the reaction was stopped by injecting 0.5 ml of 10 % TCA. The tubes were re-incubated for an additional 30 min to trap all liberated $^{14}CO_2$. The center wells were removed and placed in scintillation vials containing 10 ml of scintillation mixture (4.0 g PPO ; 0.2 g POPOP in 1 litre of toluene). The samples were counted and the enzyme activity is given as pmoles of CO_2 liberated per mg protein per hour. Protein was estimated as per the method of Lowry et al (11).

Incorporation of ^{3}H-leucine into TCA precipitable proteins was determined at 4 hours after injection of ^{3}H-leucine (5 µCi per testis). The precipitated protein was washed twice with 5 % TCA, dissolved in 0.5 ml of Beckman tissue solubilizer and counted in toluene based scintillant using a Beckman Liquid Scintillation Spectrometer (Model LS 3133 P).

RESULTS

Fig.1 shows the effect of PGE_2(10 µg/testis) on the activity of CDC at various time intervals. The enzyme activity increased significantly at 1 hr ($P= 0.05$) reaching maximal levels at 2 hrs ($P < 0.01$) after the injection. This was followed by significant reduction at 4 hrs ($P<0.01$) declining to control level at 6 hrs.

The effect of different doses of PGE_2 on CDC activity is shown in Fig.2. An increase in the activity of the enzyme was observed following treatment with 5 µg of PGE_2, reaching maximal levels at a dose of 10 µg per testis. Higher doses of PGE_2 caused significantly smaller stimulation in the activity of the enzyme.

The effect of PGE_2 and $PGF_{2\alpha}$ alone and PGE_2 in combination with cAMP or MIX is given in table 1. Injection of MIX to prostaglandin treated animals caused additional stimulation of CDC activity when compared with the animals injected with MIX or PGE_2 alone. cAMP caused an increase in the activity of CDC ($P<0.01$). Injection of PGE_2 to cAMP treated animals induced additional stimulation of the enzyme. $PGF_{2\alpha}$ did not cause enhanced stimulation of CDC in the PGE_2 injected animals.

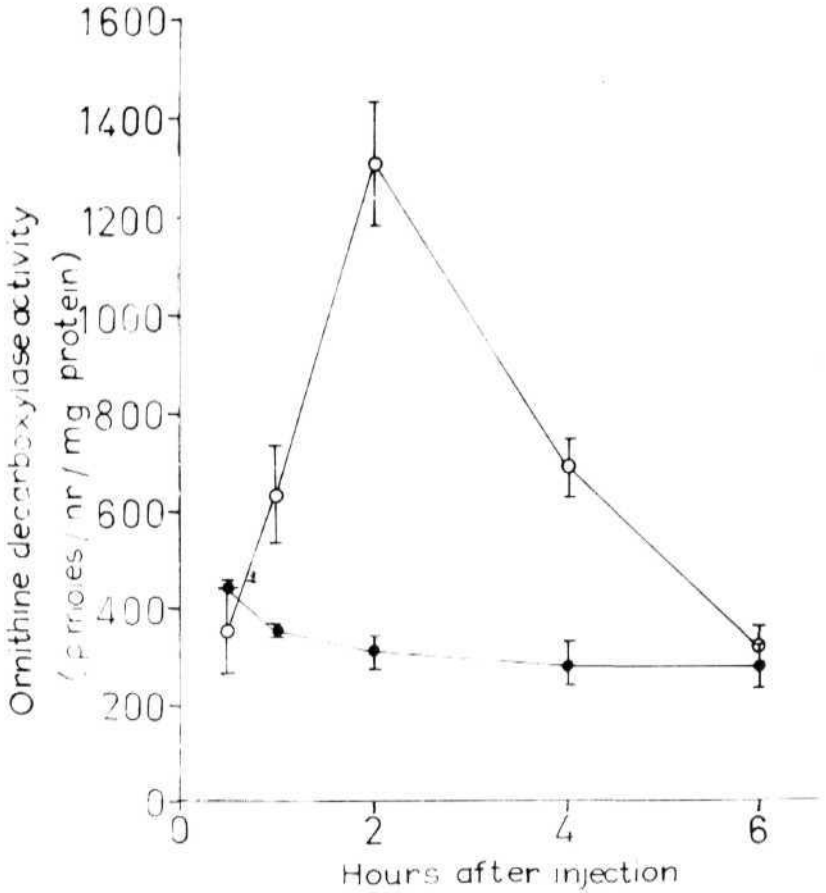

Fig. 1. Effect of PGE_2 on the levels of ODC activity in the testis of rat at various time intervals. PGE_2 was injected at a dose of 10 µg per testis. Each point is a mean ± S.E.M. of 6-10 animals. O——O PGE_2, ●— — ● saline

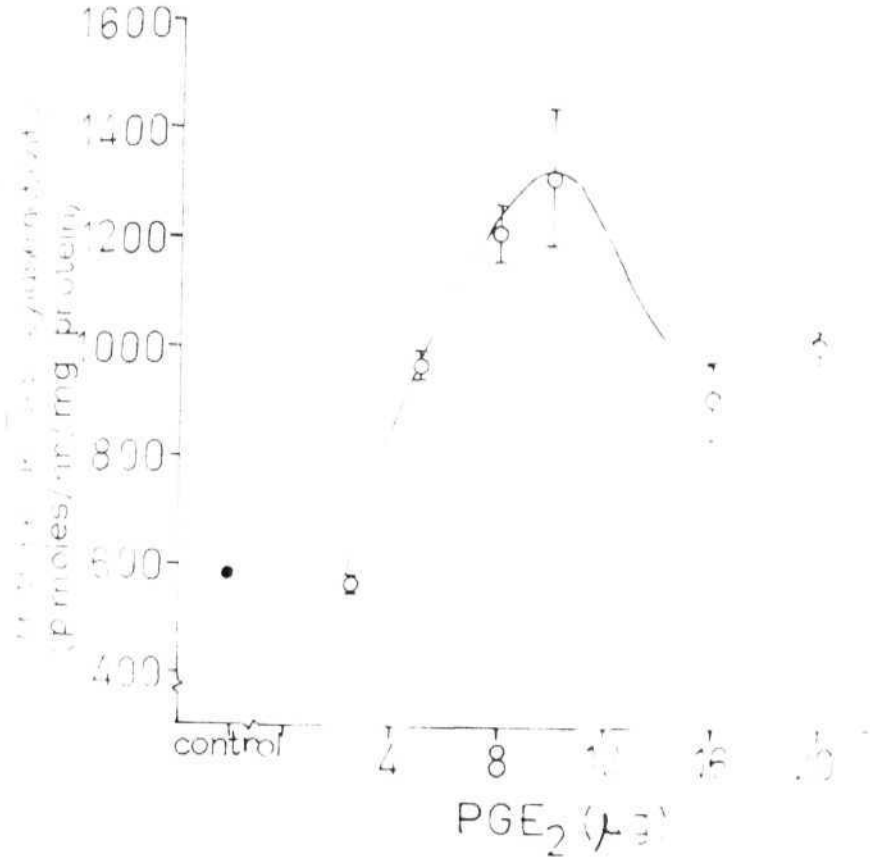

Fig. 2. Effect of various doses of PGE_2 on ODC activity of immature rat testis. PGE_2 was injected intratesticularly and the enzyme activity was determined at 2 hrs. as described in materials and methods section. Each point is a mean ± S.E.M. of 6-10 animals.

TABLE 1. EFFECT OP PGE_2, $PGF_{2\alpha}$, cAMP AND MIX ON ORNITHI DECARBOXYLASE ACTIVITY IN TESTIS

Groups	Treatment	ODC activity pmoles/hr/mg protein	
1.	Control	455 ± 9	
2.	PGE_2(10µg)	1315 ± 123	
3.	$PGF_{2\alpha}$(10µg)	1152 ± 24	
4.	PGE_2 (10µg)+$PGF_{2\alpha}$(10µg)	950 ± 125	
5.	cAMP (20µg)	726 ± 55	$p < 0.01$
6.	PGE_2(10µg)+cAMP(20µg)	997 ± 51	
7.	MIX (5µg)	804 ± 61	$p < 0.001$
8.	MIX (5µg) +PGE_2(10µg)	1902 ± 129	

Prostaglandins, MIX and cAMP were injected directly into the testis in 5-10 µl of vehicle as described in materials and methods section and the animals were killed after 2 hra. Results are mean ± S.E.M. of 6-10 animals per group. Group 8 is significantly different from group 2 ($p < 0.05$)

The effects of LH, FSH individually or in combination with PGE_2 is given in table 2. The gonadotropic hormones caused significant stimulation of ODC activity at a dose of 20 µg per testis. PGE_2 at a dose of 5 or 10 µg per testis to the LH or FSH Treated animals caused additional stimulation of ODC activity.

The effect of indomethacin, potent inhibitor of prostaglandin synthetase, on LH, FSH or cAMP stimulated ODC activity was studied (Table 3). Indomethacin caused drastic inhibition of ODC activity in FSH, LH or cAMP treated groups.

3 2. EFFECT OF COMBINED TREATMENT WITH PGE_2 AND GONADGTROPIC HORMONES ON ODC ACTIVITY IN THE TESTI3

	Treatment	ODC activity (p moles/hr/mg protein)	
1.	Control	455 ± 9	
2,	PGE_2(10µg)	1315 ±125	
3.	PGE_2(5µg)	969 ± 15	p<0.001
i.	LH (20µg)+PGE_2(5µg)	1527 ± 123	
5.	LH (20µg)	1354 ± 34	p<0.001
6.	LH (20µg)+PGE_2 (10 µg)	2082 ± 93	
7.	FSH (20µg)	793 ± 42	p<0.01
3.	FSH (20µg)+PGE_2 (5µg)	1125 ± 71	

Hormones and PGE_2 were administered intratesticularly in 5-10 µl of vehicle as described in materials and methods section. Animals were killed 2 hrs. after the injection. Results are mean ± S.E.M. of 6-10 animals per group.

To assess the possibility that indomethacin was acting at the level of general protein synthesis, an experiment was conducted using ^{3}H-leucine. It was observed that FSH induced leucine incorporation was drastically inhibited by 30 or 60 µg of indomethacin per testis(p<0.001). The results are given in table 4.

TABLE 3. EFFECT OF INDOMETHACIN ON FSH, LH AND cAMP STIMULATED ODC ACTIVITY

Treatment	ODC activity (pmoles/hr/mg protein)	Inhibition (percent)
1. Control	597 ± 14	
2. IM (60µg)	538 ± 5	
3. PSH (40µg)	1456 ± 182	
4. FSH (40µg)+IM (30µg)	1214 ± 180	28
5. FSH (40µg)+IM (60µg)	549 ± 70	100
6. LH (40µg)	1224 ± 134	
7. LH (40µg)+IM (60µg)	609 ± 88	98
8. cAMP (40µg)	879 ± 17	
9. cAMP (40µg)+IM (60µg)	537 ± 93	100

Hormones and cAMP were injected in 10µl of saline. Indomethacin was injected in 5 µl of ethanol. Animals in groups 1-7 were killed at 4 hrs. and in groups 8 and 9 were killed 2 hrs. after the injection. Results are mean + S.E.M. of 6-10 animals. Injection of 5 µl of ethanol alone to FSH treated animals (data not shown) did not cause any inhibition of ODC activity. Inhibition of 28% observed in group 4 is not statistically significant when compared to group 3 ($p>0.4$).

TABLE 4. EFFECT OF INDOMETHACIN ON ^{3}H-LEUCINE INCORPORATION INTO PROTEINS OF TESTIS TREATED WITH F3H

	Treatment		^{3}H-leucine incorporated (cpm/mg protein)
1.	Control (Saline)	(6)	66,900 ± 8900
2.	IM (60 ug)	(4)	54,800 ± 7600
3.	FSH (40 µg)	(4)	115,200 ± 6700
4.	FSH (40 µg)+IM (30 ug)	(4)	46,500 ± 2200
5.	FSH (40 ug) +IM (60 µg)	(4)	39,800 ± 3000

The number of animals used in each group is given in parentheses. The values are mean ± S.E.M.

DISCUSSION

The present study shows that PGE_2 and $PGF_{2\alpha}$ like FSH, LH or cAMP, cause stimulation of ODC activity in the testis of rat. These results are similar to the observations made earlier on the effect of PGE_2 on rat ovary in vivo (7) and in vitro (6). It was interesting to observe that in our studies treatment with FSH or LH along with the maximally effective dose of PGE_2 (10µg/testis) caused additional stimulation of ODC activity. The levels of cAMP in the testis increase following treatment with gonadotropic hormones (12,13) and prostaglandins (4,14). It was shown that stimulation of ODC activity by gonadotropic hormones is mediated by cAMP in porcine granuloea cells (15). Hyperstimulation of ODC activity observed in the present study following treatment with saturating levels of PGE_2 and FSH or PGE_2 and LH may be due to the additional accumulation of cAMP due to the combined action of these compounds. It is also possible that PGE_2, LH and FSH are acting on different cell types and are causing additional ODC activity. Studies using isolated cell types are necessary to investigate this possibility. $PGF_{2\alpha}$ in combination with PGE_2 did not cause any additive effect. This may be due to the action of these compounds on similar types of cells in the testis. The idea that cAMP mediates the

stimulation of ODC activity is strengthened in our experiments using phosphodiesterase inhibitor, MIX. We observed that MIX potentiated the effect of PGE_2 and caused additional stimulation of ODC activity.[2] However, treatment with PGE_2 and cAMP did not stimulate the activity of ODC over the levels of PGE_2 injected animals probably due to rapid destruction of cAMP.

The intermediary role of prostaglandins in LH or FSH action is not clear. Treatment of LH or FSH injected animals with indomethacin, a prostaglandin synthetase inhibitor caused inhibition of ODC activity. However, similar inhibition of ODC activity by indomethacin was also observed in cAMP treated animals. Indomethacin appears to have toxic effect on testis since it inhibited incorporation of ^{3}H-leucine into total proteins, in addition to causing inhibition of ODC activity. Osterman *et al* (16) have also found that indomethacin caused inhibition of ODC activity and protein synthesis in the granulosa cells of ovary *in vitro*. A more specific inhibitor of prostaglandin synthetase which is less toxic to the tissue is necessary to study the role of prostaglandins in gonadotropic hormone action.

ACKNOWLEDGEMENTS

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EFFECT OF CATECHOLAMINES ON ORNITHINE DECARBOXYLASE ACTIVITY IN THE TESTIS OF IMMATURE RAT

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SUMMARY

Intratesticular injection of epinephrine and norepinephrine caused stimulation of ornithine decarboxylase (ODC) activity in the testis of immature rat. The effect of epinephrine was time and dose dependant. The minimal effective dose for epinephrine was found to be 100 pg and optimal stimulation was observed with 500 ng of the drug. Maximal stimulation of ODC occurred at 2 h after the treatment and reduced significantly at 4 h reaching to control levels at 6 h. Simultaneous injection of epinephrine with dibutyryl cAMP, luteinizing hormone, follicle stimulating hormone or prostaglandin E_2 caused additional stimulation of the enzyme activity. Injection of epinephrine to norepinephrine treated animals caused additional effect. Both epinephrine and norepinephrine were found to stimulate the enzyme activity in Leydig cell and seminiferous tubule fractions. These results suggest that catecholamines are also involved in the regulation of ODC activity in the testis of rat.

INTRODUCTION

Several recent reports indicate that factors other than gonadotropic hormones may influence testicular function. The testes of rat contains adrenergic nerve terminals in close proximity to testicular blood vessels (1). Large doses of epinephrine and norepinephrine affect testicular vasoconstriction and blood flow (2,3). It was observed that the general metabolic activity of the testis is altered by the addition of epinephrine in vitro to the testicular tissue (4)

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and injection of catecholamines caused reduction in the levels of testosterone (5). However, the levels of cAMP were found to increase in the fiertoli cells following treatment with catecholamines in vitro (6). Ornithine decarboxylase (EC 4.1.1.17) is a rate limiting enzyme in the biosynthesis of putrescine and polyamines (7). In our earlier studies we have reported that the levels of testicular ODC are regulated by gonadotropic hormones and prostaglandins, probably through the stimulation of cAMP (8-10). The present study, for the first time, shows that epinephrine and norepinephrine stimulate the levels of ODC in the testis of rat.

MATERIALS AND METHODS

Immature male rats, aged 21-22 days, weighing between 25-30 g, derived from Wistar strain were used in this study. Ovine luteinizing hormone (NIH-LH-S-20), ovine follicle stimulating hormone (NIH-FSH-S-12) were generously provided by the National Pituitary Agency, NIAMDD, U.S.A. L-epinephrine bitartarate, L-norepinephrine bitartarate, pyridoxal phosphate, ornithine, dithiothreitol, glutathione and dibutyryl cyclic AMP were purchased from Sigma Chemical Company. Prostaglandin E_2 (PGE_2) was obtained from Upjohn Company. D,L-(1-^{14}C) ornithine monochloride (58 mCi/mmol) was obtained from Radiochemical Centre, England. All other chemicals were of analytical grade and were procured locally.

The animals were injected intratesticularly under mild ether anesthesia with hormones, PGE_2 or cAMP in 5-10 µl of 0.15M sodium chloride as described previously (8). At appropriate time the animals were killed by cervical dislocation and the decapsulated testes were homogenized in 4 vol of ice cold TED buffer (Tris, 25mM, EDTA 0.1mM, DTT 1.0mM, pH 7.4). The homogenate was centrifuged at 25,000 g in a MSE refrigerated centrifuge for 30 min. The supernatant was used for the assay of ODC activity essentially according to the method of Janne and Williams-Ashman (11) with some modifications as described earlier (9). Leydig cells and seminiferous tubules were separated by incubation in Krebs-Ringer bicarbonate buffer, pH 7.4, containing 1 mg/ml collagenase and the ODC activity of the separated cells was estimated as described by us (10). Protein content was determined by the method of Lowry et al (12).

RESULTS

The effect of 1 µg epinephrine on total testicular ODC activity at various time intervals is shown in Fig. 1. It

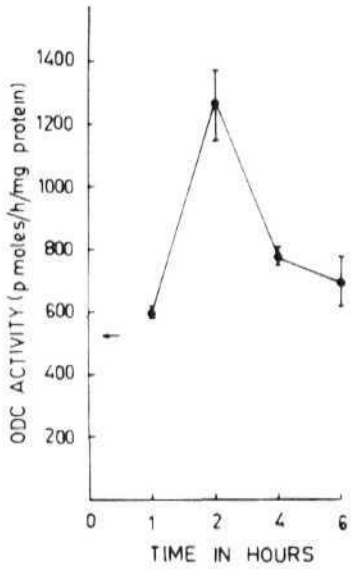

Fig. 1 Time course of action of epinephrine on ODC activity in the testis. Epinephrine at a dose of 1µg/testis was injected intrate6ticularly and the animals were killed at various time intervals. The arrow indicates the untreated control value.

was observed that epinephrine caused maximal stimulation of ODC at 2 h after the injection of epinephrine. The enzyme activity declined at 4 h and returned to control levels at 6 h. Table I shows the effect of various doses of epinephrine and norepinephrine on ODC activity at 2 h after the injection. Both epinephrine and norepinephrine caused significant stimulation of the enzyme activity following injection with 100 pg of the drug. However, injection with less amount of the catecholamines did not cause any effect (data not shown). Maximal stimulation of the enzyme activity was observed with 500 µg of epinephrine and 1 µg of norepinephrine. The effect of catecholamines in the Leydig cell and seminiferous tubule fractions is given in table II. Epinephrine and norepinephrine injection caused significant stimulation of ODC activity in both Leydig cells and seminiferous tubules.

Table 1

EFFECT OF VARIOUS DOSES OF EPINEPHRINE AND NOREPINEPHRINE ON ODC ACTIVITY IN THE TESTIS

Treatment	Dose/Testis	ODC activity (p moles/h/mg protein)
Saline	----	709 ±112
Norepinephrine	100 pg	1087 ± 51
	10 ng	1047 ± 33
	100 ng	1197 ± 94
	1.0 µg	1419 ± 19
	10.0 µg	1628 ± 140
Epinephrine	100 pg	1083 ± 43
	500 ng	1353 ± 113
	1.0 µg	1268 ± 114
	10.0 µg	1357 ± 121

All treated groups were statistically significant ($P<0.05$ – $P<0.001$) when compared to the saline treated controls. 6-10 animals were used in each group. ODC activity was measured at 2 h after the injection of the compounds.

Table III shows the effect of combined treatment of epinephrine and FSH, LH, PGE_2, norepinephrine or cAMP on ODC activity at 2 h after the injection. It was observed that simultaneous injection of any of the above compounds to epinephrine treated animals caused significantly higher stimulation of the enzyme activity.

DISCUSSION

These results show that both epinephrine and norepinephrine cause stimulation of ODC activity. The enzyme activity

Table II

EFFECT OF EPINEPHRINE AND NOREPINEPHRINE ON ODC ACTIVITY IN THE ISOLATED LEYDIG CELLS AND SEMINIFEROUS TUBULES

Treatment	ODC activity (p moles/h/mg Drotein)	
	Leydig cells	Seminiferous tubules
Control	322 ± 18	549 ± 40
Epinephrine, 1 µg	678 ± 101	999 ± 96
Norepinephrine, 1 µg	754 ± 43	1064 ± 34

Each group consists of 3-4 determinations containing 15-20 animals. All treated values are significantly ($P<0.001$) different from controls. ODC activity was measured at 2 h after the injection of catecholamines.

was dose and time dependant. Furthermore it is interesting to note that the catecholamines increase the enzyme activity in both Leydig cells and seminiferous tubules. In an earlier study we have observed that PGE_2 and $PGF_{2\alpha}$ also cause stimulation of ODC activity in Leydig cells and seminiferous tubules, while the action of FSH is confined to seminiferous tubules and that of LH to Leydig cells (10).

It is interesting to note that injection of norepinephrine, PGE_2, LH, FSH or cAMP to the animals treated with saturating dose of epinephrine causes additional stimulation of ODC activity. This may be due to the action of these compounds on different types of cells causing hyperstimulation of ODC activity in the whole testis. In our previous study it was observed that injection of PGE_2 to LH or FSH treated animals also caused additional stimulation of ODC activity. Additional stimulation of ODC activity observed in this study

Table III

COMBINED EFFECT OF CATECHOLAMINES, GONADOTROPIC HORMONES, PGE_2 AND cAMP ON ODC ACTIVITY IN THE TESTIS

Group No.	Treatment	ODC activity (p moles/h/mg protein)	Comments
1.	Control	709 ± 64	
2.	Eplnephrine (1µg)	1268 ± 114	P<0.01 vs group 1
3.	Norepinephrine (1µg)	1419 ± 19	P<0.001 vs group 1
4.	Epinephrine (1µg) + Norepinephrine (1µg)	2328 ± 230	P<0.01 vs group 2
5.	Epinephrine (1µg) + cAMP (20µg)	1870 ± 143	P<0.05 vs group 2
6.	Epinephrine (1µg) + LH (40µg)	1772 ± 142	P<0.05 vs group 2
7.	Epinephrine (1µg) + FSH (40µg)	1992 ± 87	P<0.01 vs group 2
8.	Epinephrine (1µg) + PGE_2 (10µg)	2188 ± 106	P<0.001 vs group 2

6-10 animals were used per group. ODC activity was estimated at 2 h after the injection of the various compounds.

following treatment with norepinephrine to epinephrine treated animals is interesting. This may be due to the action of these two catecholamines on two different types of receptors on the same cell or due to their action on entirely different Populations of the cells in the testis. Furthermore, PGE_2 which also acts on both seminiferous tubules and Leydig cells also caused additional stimulation in animals treated with saturating dose of epinephrine. These results support the hypothesis that hyperstimulation of ODC activity in these animals is due to their action on different types of cells.

Catecholamines were shown to increase the activity of ODC in porcine granulose cells (13). It was proposed that

in rat granulasa cells the effect may be mediated by β-2-receptors linked to adenylate cyclase system (13). In rat Sertoli cells β-1-adrenergic receptors were identified and catecholamines were shown to increase the levels of cAMP (6). It is possible that the increase of CDC following injection with epinephrine and norepinephrine is due to the increase in the levels of intracellular cAMP. It was proposed earlier that the increase in the levels of CDC following injection with LH, FSH, PGE_2 and $PGF_{2\alpha}$ is mediated through cAMP in the testis of rat (8-10). Additional stimulation of CDC observed in this study in response to epinephrine and LH, FSH, PGE_2 or norepinephrine may be due to the increased stimulation of cAMP due to the combined action of these agents. However, further work is necessary to elucidate the mechanism of action of catecholamines and their physiological role in the testis.

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STIMULATION OF ORNITHINE DECARBOXYLASE ACTIVITY BY PROSTAGLANDINS IN THE ISOLATED CELLS OF IMMATURE RAT TESTIS

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1. Introduction

In [1] evidence was presented showing hyperstimulation of ornithine decarboxylase (ODC) activity following combined treatment with saturating levels of prostaglandin F2 (PGE_2) and follicle-stimulating hormone (FSH) or PGE_2 and luteinizing hormone (LH) in the testis of immature rat [1]. It was presumed that PGE_2, FSH and LH were probably acting on different cell types and were causing a cumulative effect on ODC. Hence experiments were designed to study the effect of prostaglaridins. FSH and LH on ODC activity in the isolated Leydig cells and seminiferous tubules of immature rat testis. The evidence presented in this study shows that PGE_2 and $PGF_{2\alpha}$ stimulate ODC levels in both Leydig cells and seminiferous tubules.

2. Materials and methods

Ovine luteinizing hormone (NIH-LH-S-20), ovine follicle stimulating hormone (NIH-FSH-S-12) and prostaglaridins were obtained from the National Institutes of Health and the Upjohn Company, respectively. Ornithine, pyridoxal phosphate, dithiothreitol, reduced glutathione, collagenase and bovine serum albumin were purchased from Sigma. St Louis. D,L-[1-^{14}C] ornithine monochloride (58 mCi/mmol) was purchased from the Radiochemical Centre, Amersham. All other chemicals were of analytical grade and were obtained locally.

Stock solutions of prostaglaridins were prepared in ethanol at 10 mg/ml. They were diluted with saline to the desired concentrations before injecting the animals. Hormones were made in saline.

Immature male rats (21–22 days. 25–30 g) were used in all experiments. Rats were injected intratesticularly with 10 μg PGE_2 alone or in combination with 40μg FSH or LH in 10 μl total vol. as in [2]. Control animals received 10 μl saline. At appropriate times rats were killed by spinal dislocation and testes were dissected out. The decapsulated testes from 6–8 animals were pooled and Leydig cells and seminiferous tubules were separated by incubation in Krebs-Ringer bicarbonate buffer (pH 7.4) containing collagenase (1 mg/ml) as in [3]. Addition of 1 mM reduced glutathione increased the stability of ODC, hence it was added to the buffer during incuhation.

The Leydig cells and seminiferous tubules, following their separation, were homogenized in 4 vol. 25 mM Tris, 0.1 mM EDTA and 1 mM DTT buffer (pH 7.4) in a glass homogenizer, and centrifuged at 25 000 X *g* for 30 min. The supernatant was used for the assay of ODC activity as detailed in [4]. The assay mixture contained 0.25 μmol unlabelled ornitiinc, 2.5 μmol DTT, 0.1 μmol pyridoxal phosphate, 0.2 μCi radioactive ornithine and 200 μl enzyme in 0.5 ml total vol. Following incubation at 37°C for 1 h the reaction was stopped by injecting 0.5 ml 10% trichloroacetic acid and the tubes were re-incubated for an additional 30 min to trap all liberated $^{14}CO_2$ in the centre wells which contained 0.1 ml hyamine hydroxide. The radioactivity was counted in a toluene-based scintillant using a Beckman Liquid Scintillation Spectrometer (Model LS 3133 P). Protein was determined by the Lowry method [5]. ODC activity is expressed as pmol $^{14}CO_2$ liberated . h^{-1}. mg $protein^{-1}$.

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3. Results and discussion

The effects of PGE_2 and $PGF_{2\alpha}$ alone and PGE_2 in combination with FSH or LU are shown in table 1. It was observed that PGE_2 and $PGF_{2\alpha}$ significantly stimulated the activity of ODC in both Leydig cells and seminiferous tubules. LH caused significant stimulation of the enzyme activity in Leydig cells while FSH increased the levels in seminiferous tubules.

The above results show that PGE_2 and $PGF_{2\alpha}$ act on both Leydig cells and seminiferous tubules and stimulate ODC activity. Treatment with FSH and PGE_2 did not increase the levels of ODC in the seminiferous tubules over the levels seen in animals treated with PGE_2 alone. Similarly injection of LH and PGE_2 did not cause hyperstimulation in the Leydig cell fraction. This may be due to a common rate-limiting intermediate in the action of both these compounds Cyclic AMP stimulated the levels of ODC in [1,2]. Prostaglandins [6J and gonadotropic hormones [7] are known to stimulate cAMP levels in the testis of rat. Stimulation of ODC levels observed here following treatment with prostaglandins and gonadotropic hormones may be through the stimulation of cAMP levels in the Leydig cells and seminiferous tubules of rat. Hyper-stimulation of ODC levels following treatnieni with PGE_2 and gonadotropic hormones observed earlier in the whole testis [1] appears to be due to the combined effect of these compounds on different cell types in the testis of rat.

Table 1
Effect of PGE_2, $PGF_{2\alpha}$, FSH and LH on ODC activity in the isolated leydig cells and seminiferous tubules of testis

Croup no.	Treatment	ODC (pmol . h^{-1} . mg protein^{-1})	
		Leydig cells	Seminiferous tubules
1.	Saline	322 ' 18	549 - 40
2.	PGE_2	970 > 133[d]	1852 J. 228[d]
3.	LH	540 ' 75[a]	64 3 > 69
4.	LH + PGE_2	859 ' 79[d]	1116 ± 176[b]
5.	FSH	360 > 46	1006 - 176[a]
6.	FSH + PGE_2	75 3 ' 29 2[a]	1033 ' 144[c]
7.	$PGF_{2\alpha}$	1012 > 110[d]	1277 ± 338[c]

[a] P •: 0.05; [b] P •:: 0.02; [c] P < 0.01; [d] P < 0.001 as compared to group 1

All animals were killed at 2 h alter the injection of prostaglandins or at 4 h after the injection of LH or FSH. The animals in groups 4 and 6 were treated with PGE_2 2 h alter the administration of LU or FSH. The values are mean ± SEM of 4 – 5 different observations

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