Interaction of Prostaglandin E_1 with Specific Receptors in Rat Leydig Cells

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Abstract. The Scatchard plot analysis of the binding of ^{3}H—PGE_1 to Leydig cells of rat testes revealed heterogeneity of the binding sites. Kinetic studies indicated that such a heterogeneity is not caused by negative cooperativity but is rather due to the presence of different binding sites characterized by $Ka_1 = 2.2 \times 10^8$ mol^{-1} l and $Ka_2 = 8.7 \times 10^7$ mol^{-1} l. The specific PGE_1 binding increased up to the 50th postnatal day and then decreased gradually. The changes in the binding capacity of Leydig cells did not correlate with the responsiveness to PGE_1 in cAMP and testosterone production. The highest responsiveness of Leydig cells to PGE_1 in cAMP synthesis was found during the first postnatal month, in testosterone synthesis at the age of 50 days.

Seven-day treatment of rats with 30 IU PMSG, 100 IU hCG, 1 mg testosterone and 100 μg estradiol caused a reduction in ^{3}H—PGE_1 binding to Leydig cells. One mg of cortisol applied for seven days had a stimulatory effect, whereas 6 IU of ACTH did not change the PGE_1 binding.

Plasma levels of testosterone were increased after long-term administration of PMSG and hCG, remained unchanged in ACTH and cortisol treated rats, and were markedly reduced in animals injected with estradiol.

Key words: Leydig cells — PGE_1 receptors — cAMP — Steroidogenesis

Introduction

The ability of prostaglandins (PG) to affect gonadal cAMP production and steroidogenesis was first recognized in the ovary (Marsh 1970; Kuehl et al. 1970; Kolena and Channing 1972). It has been demonstrated that PG are synthesized and metabolized also in the testicular tissue (Carpenter et al. 1978). Prostaglandins can modify the function of the testes. Saksena et al. (1978) reported a decrease of plasma testosterone levels after in vivo administration of PGE to rats. On the other hand, Eik-Nes (1969) found an increse of testosterone secretion in the dog testis perfused with PGE_2. The stimulatory effect of prostaglandins on the secretion of testosterone in vivo was also observed in monkeys (Kimball et al. 1979) and in vitro in the interstitial tissue of the rat (Sairam 1979; Šeböková and Kolena 1978).

These results are in agreement with the stimulatory effect of PGE_1 and PGE_2 on the production of cAMP observed either in isolated Leydig cells (Cooke et al. 1974; Šeböková and Kolena 1978, 1981) or in the whole rat testis (Kolena 1975).

It has been demonstrated that the stimulatory effects of PGE_1 are mediated by specific PGE_1 receptors in rat Leydig cells (Šeböková and Kolena 1978).

The binding of prostaglandins was found to be dependent on the functional state of the ovarian tissue (Šeböková and Kolena 1979) and can be modulated by hormones (Rao 1975). The aim of the present investigation was to study the regulation of PGE_1 receptors in rat Leydig cells and to estimate the relationship between PGE_1 receptors, cAMP production and steroidogenesis during changed gonadal function in the rat.

Material and Methods

Male Wistar rats of a specific pathogen free colony bred in our institute were used.

The rats of various ages (25-to 120-days-old) were sacrificed by decapitation and the testes were immediately removed, decapsulated and weighed.

Leydig cell preparations were obtained by collagenase treatment of the rat testis according to the method of Janszen et al. (1976). Concisely, the testes (about 1500 mg of tissue) were incubated in 2 ml of basal EAGLE's medium (BME) containing 0.1% bovine serum albumine (BSA) at pH 7.0 and 0.025% collagenase at 34 °C for 20 min. After the incubation ten volumes of TS buffer (0.01 mol/l Tris, 0.15 mol/l NaCl) pH 7.4 were added. The tubes were inverted several times and then left for 10 min at room temperature. The supernatant was filtered through a 80 μm nylon gauze and centrifuged at $150 \times g$ for 15 min. The sediment was washed again and then resuspended in the incubation buffer.

The effect of hormones was investigated in 25-day-old rats after seven daily i.m. injections of one of the folloving preparations: 30 IU pregnant mare serum gonadotropin PMSG (Gestyl, Organon, Holland), 100 IU of human chorion gonadotropin hCG (Praedyn, Spofa, ČSSR), 6 IU of adrenocorticotropic hormone ACTH (Cortrophine-Z, Organon, Holland), 1 mg of cortisol F (Hydrocortisone, les laboratories Roussel, Paris, France), 1 mg of testosterone T (Agovirin, Biotika-Slovenská Lupča, ČSSR), dissolved in 0.1 ml of saline. The last injection was applied 2 days before the aminals were sacrificed, except for the cortisol-treated rats which were sacrificed one day after the last injection. The control group of animals received only saline injections. Blood was collected in heparinized tubes, centrifuged, and the plasma was stored at −20 °C until assayed for testosterone.

^3H—PGE_1 binding studies were carried out on using an in vitro system (Šeböková and Kolena 1978).

The Leydig cell suspension (about 3.10^5 cells per 1 ml) in TS buffer (0.01 mol/l Tris, 0.15 mol/l NaCl) pH 7.4 was incubated with 1.86 kBq ^3H—PGE_1 in the absence or presence of 270 nmol/l unlabelled prostaglandin PGE_1 at 22 °C for 2 h. At the end of incubation 2 ml of ice-cold buffer were added and the tubes were centrifuged at $1500 \times g$ for 10 min at 4 °C. After withdrawal of the supernatant the pellet was washed with 3 ml of buffer. The washed pellet was solubilized in 0.5 ml of Soluene — 350 and the radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer.

The difference between ^3H—PGE_1 binding in the absence or presence of an excess of unlabelled PGE_1 indicates the specifically bound prostaglandin which is presented in our results.

cAMP production

Leydig cell suspension (about 3.10^5 cells per 1 ml) was incubated with or without 27 μmol/l PGE_1 at 37 °C for 20 min under continuous shaking in Krebs-Ringer-phosphate buffer of pH 7.2 with 6 mmol/l glucose and 7.5 mmol/l aminophyline. After incubation the cells plus medium were homogenized and centrifuged at 2500 × g for 10 min. The supernatant was stored at −20 °C for subsequent measurement of cAMP.

The levels of cAMP in 50 μl aliquots were determined by the competitive protein binding assay according to Gilman (1970), where the separation of protein-bound cAMP from free nucleotide was performed by the adsorption of unbound cAMP on dextran-coated charcoal (Kolena 1975).

Testosterone production

One ml of the Leydig cell suspension (about 3.10^5 cells) was incubated at 34 °C for 3 h in Krebs-Ringer-bicarbonate buffer of pH 7.4, with 6 mmol/l glucose in the presence or absence of 27 μmol/l PGE_1.

After homogenization of cells in the incubation medium, testosterone was extracted with 5 ml of ether, evaporated to dryness, dissolved in 0.5 ml phosphate buffer of pH 7.0 and stored at −20 °C for estimation of testosterone.

The concentration of testosterone was measured by radioimmunoassay without chromatography, using dextran-coated charcoal for the separation of the bound and free steroid (Šeböková and Kolena 1978). Antiserum against testosterone-3-carboxim-BSA was kindly supplied by Dr. J. Pícha (Research Institute of Animal Production, Praha, ČSSR). Its initial dilution was 1:15000. The cross reactions were: with dihydrotestosterone 9.6%, androstenedion 0.1%, androsterone 0.04% and epitestosterone 0.01%. The mean recovery for testosterone was 87 ± 5% and the within assay coefficient of variation was 8%.

The protein content was determined by the method of Lowry et al. (1951) using BSA as a standard.

The results are experessed as means ± SEM. Student's t-test was used for evaluating the differences between groups.

Collagenase (150 IU mg^{-1}, ÚSOL, Praha, ČSSR); BME (G—11, Gibco, USA); Adenosine 3',5'-cAMP, Sigma, St. Louis, USA; Testosterone (4-androsten-17 -ol-3-one) Koch-Light Laboratories Ltd., England; PGE_1, Chinoin, Budapest, Hungary and UPJOHN Co., Kalamazoo, USA; (8—^{3}H) Adenosine 3',5'-cyclic phosphate, spec act. 1.11 TBq mmol/l; 1,2,6,7 (n)-^{3}H-testosterone, spec. act. 3.2 TBq mmol/l, 5,6 (n)-^{3}H-PGE_1 spec. act. 2.2 TBq mmol/l Radiochemical Centre, Amersham, England; Norit A-Serva, Feinbiochemica, Heidelberg, FRG; Dextran T-70, Pharmacia, Fine Chemicals, Uppsala Sweden.

Results

In our previous studies the presence of PGE_1 binding sites, with high affinity and specificity was demonstrated in rat Leydig cells (Šeböková and Kolena 1978, 1981).

Prostaglandin E_1 binding sites in rat Leydig cells are saturable (Fig. 1). PGE_1 binding to Leydig cells expressed as a function of ^{3}H-PGE_1 added to the incubation medium rose linearly up to the concentration of about 3 pmol ml^{-1} and reached saturation at the concentration of 5 pmol ml^{-1} of labelled PGE_1. The Scatchard plot, displaying bound/free ^{3}H-PGE_1 as a function of the total prostaglandin bound

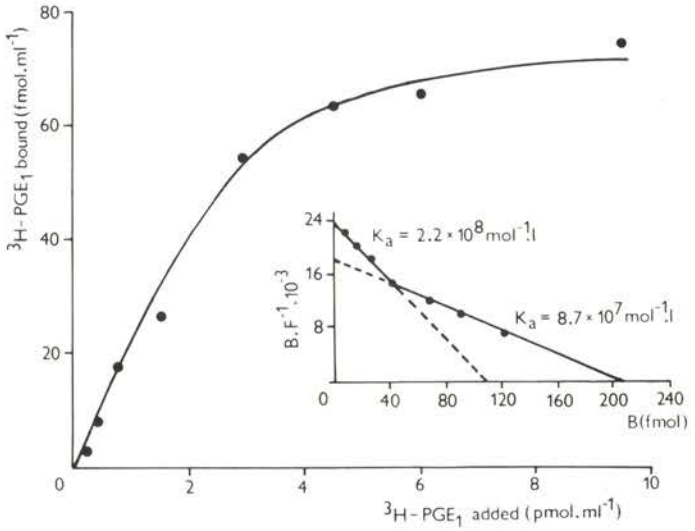

Fig. 1. The effect of increasing concentration of ^{3}H-PGE$_1$ on specific binding of ^{3}H-PGE$_1$ to Leydig cells in 52-day-old rats. The insert figure represents a Scatchard plot constructed from the data of the saturation curve. Each point represents 2 to 3 incubations.

is initially linear but curves sharply at higher binding site occupancy. The graphical resolution of this curve (as shown on insert Fig. 1) demonstrates the heterogenous nature of PGE$_1$ binding sites in rat Leydig cells. Since the value of Hill's coefficient is less than 1 ($n_H = 0.785$) the heterogeneity of ^{3}H-PGE$_1$ binding sites may be either due to the presence of independent groups of binding sites or to negative cooperativity (De Meyts et al. 1973). Which of these phenomena is involved can be demonstrated by the method based on the estimation of the dissociation rate of bound prostaglandin from its receptor at high dilution in the presence or absence of unlabelled prostaglandin. Leydig cells (in a single batch) were incubated for 2 h at 22 °C with ^{3}H-PGE$_1$ at a low concentration (0.3 kBq ml^{-1}), so that only about 10% of the receptor sites were occupied by the tracer. After washing the cells were resuspended in chilled fresh medium. An aliquot (100 µl) was removed for measurement of bound radioactivity ("0 min") and aliquots (100 µl) were transferred to series of tubes containing 10 ml of medium in the presence or absence of unlabelled prostaglandin (27 µmol/l PGE$_1$) and dissociation was followed up at 22 °C.

The bound radioactivity was plotted as a function of the time elapsed after the dilution of the system (Fig. 2).

Our results show that the dissociation of ^{3}H-PGE$_1$ from Leydig cells remains unchanged both in the absence or presence of unlabelled prostaglandin E$_1$,

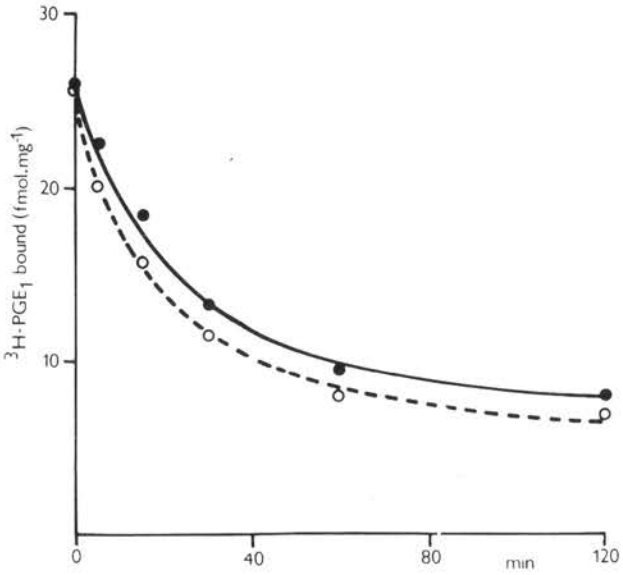

Fig. 2. Time dependence of the dissociation of specific ^{3}H-PGE$_1$ binding from Leydig cells of rat testes. Leydig cells were preincubated for 2 h at 22 °C with 0.3 kBq ^{3}H-PGE$_1$ and after washing the dissociation of bound PGE$_1$ was determined under 100-fold dilution with buffer in the absence ●——● and in the presence ○— —○ of 27 µmol/l of unlabelled PGE$_1$. Each point represents 2 to 3 incubations.

indicating that the heterogeneity of PGE$_1$ binding sites is not caused by negative cooperativity, but is rather the result of ^{3}H-PGE$_1$ binding to different and independent binding sites. The results in insert Fig. 1 show that one group of binding sites had a higher affinity and lower capacity and was characterized by the association constant $Ka_1 = 2.2 \times 10^8$ mol^{-1}l, while the other one had a lower affinity and higher capacity, $Ka_2 = 8.7 \times 10^7$ mol^{-1}l.

Prostaglandin binding sites with a specificity for PGE$_1$ have been demonstra-

Table 1. Comparison of binding characteristics of PGE$_1$ receptors in rat ovarian and testicular tissue.
(K_a = association constant)

tissue	Ka_1 mol^{-1}l	Ka_2 mol^{-1}l
ovary	2×10^8	3×10^7
testes	2.2×10^8	8.7×10^7

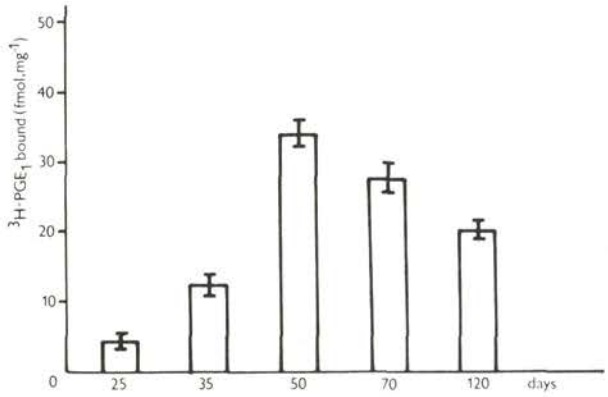

Fig. 3. Specific ^{3}H-PGE$_1$ binding to Leydig cells isolated from the testis of rats at different stages of development. Each column represents a mean ± SEM of values obtained from 4 incubations.

ted also in rat ovary slices and corpora lutea (Šeböková and Kolena, 1979). The binding constants for the interaction of ^{3}H-PGE$_1$ with ovarian and Leydig cell receptors are presented in Table 1. The association constant for high affinity PGE$_1$ testicular binding sites is comparable with that found in the rat ovary, whereas the low affinity binding site association constant is 2.5 times higher than the one calculated for ovarian tissue. The binding of ^{3}H-PGE$_1$ to Leydig cells isolated from the rat testis at various stages of development was correlated with the responsiveness of Leydig cells to PGE$_1$ in the production of cAMP and testosterone. The ^{3}H-PGE$_1$ binding capacity of Leydig cells undergoes remarkable changes from the 25th; to the 120th day of life (Fig. 3). The results show a sharp increase in prostaglandin E$_1$ binding to Leydig cells (expressed as fmol per mg of proteins) from the 25th to 50th day ($P<0.001$) followed by a gradual decrease to the 120th postnatal day ($P<0.01$).

The basal production of cAMP in Leydig cells of the rat testis was not significantly changed during the postnatal development of the rats (upper part of Fig. 4).

The stimulatory effect of PGE$_1$ on the formation of cAMP in Leydig cells was significant in each age group studied ($p<0.01$). However, the responsiveness of Leydig cells to 27 μmol/l PGE$_1$ in the synthesis of cAMP was higher in 25-, 35-and 50-day-old rats (3.8, 7.5 and 4.5 fold increase respectively) and decreased considerably in 70-and 120-day -old rats (2.3 and 1.7 fold increase, respectively).

As shown in the lower part of Fig. 4, not only the responsiveness of Leydig cells in testosterone synthesis to PGE$_1$ but also the basal production of testosterone was changing during the postnatal development of the rats. A marked increase in endogenous testosterone production was found in rats up to the 70th day of life.

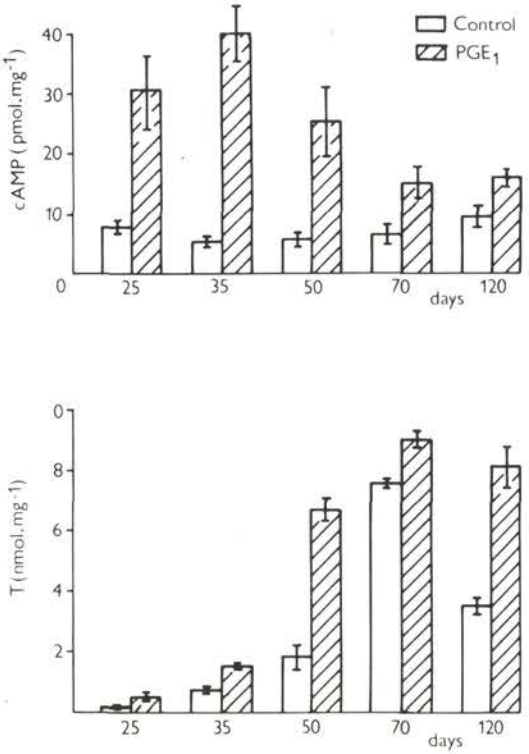

Fig. 4. Production of cAMP (upper part) and testosterone (lower part) by Leydig cells isolated from the testis at different ages in the absence (open columns) or in the presence (hatched columns) of PGE_1 (27 μmol/l). The differences in the synthesis of cAMP ($P<0.01$) and in testosterone secretion ($P<0.01$ to $P<0.001$) between control and PGE_1 stimulated samples were significant in all age groups studied. Each column represents a mean ± SEM of 3 to 4 and 4 to 6 incubations respectively.

The adult rats (120 days-old) had significantly lower testosterone secretion ($P<0.001$). PGE_1 stimulated testosterone production in Leydig cells isolated from the testis was significantly increased in all age groups ($P<0.05$ to $P<0.001$), with the highest responsiveness at the age of 50 days (3.7 fold increase).

The hormonal treatment of immature rats caused changes in ^{3}H-PGE_1 binding to isolated Leydig cells, as well as in plasma testosterone levels (Fig. 5).

The administration of PMSG and hCG to immature rats for 7 days decreased ^{3}H-PGE_1 binding in Leydig cells to 72 or 68% of the control values, respectively ($p<0.001$).

Cortisol injected into rats for 7 days had a stimulatory effect on the binding of ^{3}H-PGE_1 in rat Leydig cells (135% of control, $P<0.02$), whereas the long-term administration of ACTH had no effect on prostaglandin E_1 binding. Both steroids,

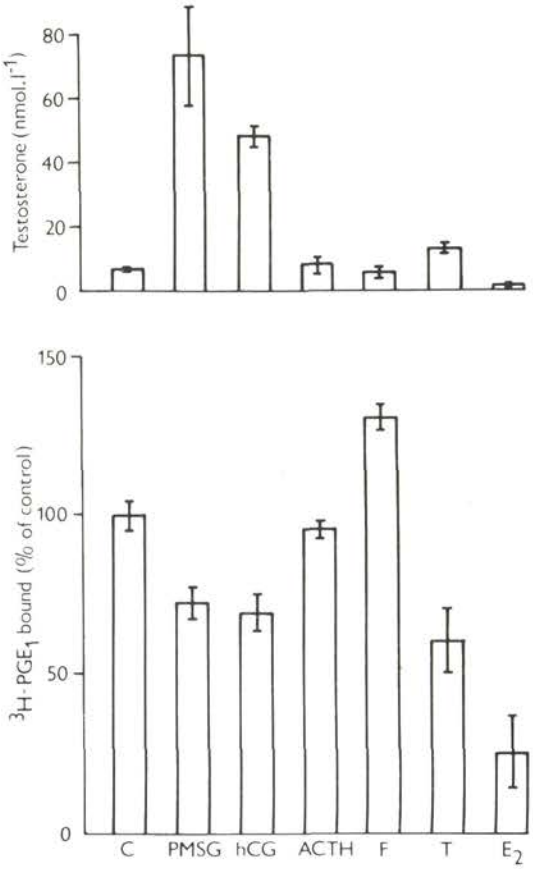

Fig. 5. The effect of daily administration of 30 IU PMSG, 100 IU hCG, 6 IU ACTH, 1 mg cortisol, 1 mg testosterone and 100 μg estradiol to immature rats on specific ^{3}H-PGE$_1$ binding to Leydig cells and plasma testosterone levels. Each column represents a mean ± SEM of 8 estimations. Statistical significance:

		^{3}H-PGE$_1$ binding	plasma testosterone
Control:	PMSG	$P<0.001$	$P<0.001$
	hCG	0.001	0.001
	ACTH	N.S.	N.S.
	cortisol	0.02	N.S.
	testosterone	0.002	0.01
	estradiol	0.001	0.001

testosterone and estradiol, repeatedly administered to rats resulted in a reduction of prostaglandin E$_1$ receptor concentration in isolated Leydig cells ($P<0.002$ and $P<0.001$).

PGE$_1$ binding to Leydig cells was reduced to 60% in testosterone-treated rats and to 25% in estradiol-injected animals as compared to controls. The

effect of the long-term action of hormones on plasma testosterone levels is shown in the upper part of Fig. 5.

PMSG and hCG treatment of rats resulted in elevated plasma testosterone levels, which were 7 to 10 times higher than in untreated animals ($P<0.001$). The administration of ACTH and cortisol did not change the concentration of testosterone, whereas estradiol caused a significant reduction in the level of testosterone from 7 ± 0.8 nmol/l as found in control animals to 1.2 ± 0.1 nmol/l ($P<0.001$).

Discussion

The data presented in this paper support our previous findings that specific PGE_1 receptors are present in rat Leydig cells (Šeböková and Kolena 1978). The Scatchard plot analysis of equilibrium binding data and Hill's coefficient ($n_H = 0.785$) indicates the heterogeneity of PGE_1 binding sites in rat Leydig cells. This heterogeneity can be caused by the presence of two groups of independent bindig sites with discrete affinities or negative cooperativity among PGE_1 binding sites. De Meyts et al. (1973) described a simple kinetic method to assess the presence of site-site interactions between insulin receptors. The same method has been recently applied to other receptors and found to be useful in detecting the presence of negative cooperativity (Limbird et al. 1975; Frazier et al. 1974).

If negative cooperativity exists among receptors, the dissociation of a hormone will be much higher in samples containing an excess of the unlabelled ligand in the dilution medium than in the tubes without it. This may be due to the occupancy of free binding sites by an excess of the unlabelled ligand, which results in lowering the affinity of the other binding sites. As a result of the lowered affinity, the bound labelled ligand will be released much faster from the receptor sites. In the absence of negative cooperativity, the dissociation curve of the bound labelled ligand will be similar both in the presence and absence of the unlabelled ligand. It is however difficult to obtain a direct proof for cooperativity between receptors and most of the equilibrium or kinetic experiments fail to distinguish which one of the various possibilities is involved.

Our experiments demonstrate that the presence of an excess of unlabelled prostaglandin E_1 in a dilution medium has no effect on the rate of dissociation of bound ^{3}H-PGE_1 from Leydig cell receptors.

These results indicate that the heterogeneity of ^{3}H-PGE_1 binding to rat Leydig cells is not caused by negative cooperativity but is rather due to the presence of two groups of independent binding sites with discrete affinities which are characterized by the association constants $Ka_1 = 2.2 \times 10^8$ mol^{-1}l and $Ka_2 = 8.7 \times 10^7$ mol^{-1}l.

The actual functional significance of these two groups of binding sites for PGE_1 in rat Leydig cells is not yet understood. The binding characteristics of PGE_1

receptors in rat Leydig cells are similar to those found in the rat ovaries (Šeböková and Kolena 1979).

It is an accepted fact that the action of prostaglandins on steroidogenesis is mediated via the formation of cAMP (Marsh 1970; Kolena and Channing 1972). The binding of prostaglandin E_1 to rat Leydig cells was shown to undergo some variations depending on the age of the animals.

The physiological state characterized by conspicuous changes in the function of the rat testes represents a suitable model for studying the relationship of PGE_1 receptors in respect to changes in cAMP and steroidogenesis. The marked increase in ^{3}H-PGE_1 binding to Leydig cells observed during sexual maturation of rats is in correlation with plasma testosterone concentration which increased to the day 60th and then gradually decreased. A similar increase in rat testicular function during early postnatal development was observed also by others (Brown-Grant et al. 1975; Knorr et al. 1970; Pahnke et al. 1975). The number of Leydig cells per testis was also reported to reach a maximum on the 50th postnatal day (Pahnke et al. 1975).

Leydig cells are under the control of pituitary gonadotropins (FSH and LH) and this effect was found to be pronounced from the neonatal period to the adult age (Purvis and Hansson 1978). The plasma concentration of FSH is high up to the 50th postnatal day and decreased later on, while the LH levels are low and remain practically unchanged.

FSH was found to increase the formation of new LH/hCG receptors in desenzitized rat testes (Kolena and Šeböková 1982). Prostaglandin receptors may be under a similar stimulatory action of FSH.

The addition of PGE_1 in vitro to Leydig cells stimulated the synthesis of cAMP and the endogenous production of testosterone. However the observed stimulatory effect of prostaglandin on cAMP and testosterone production did not correlate with the pattern of ^{3}H-PGE_1 binding sites in rat Leydig cells during postnatal development. Along with the increased PGE_1 receptor concentration in Leydig cells observed up to the 50th postnatel day, there was also an enhanced sensitivity of Leydig cells to PGE_1 in cAMP production.

It has been established that Leydig cell function is under the control of several hormones. In addition to the presence of LH receptors on Leydig cell membranes (Catt et al. 1974), receptors for androgens (Sar et al. 1975), estrogens (Mulder et al. 1974) glucocorticoids (Evain et al. 1976) and prolactin (Charreau et al. 1977) have also been demonstrated, suggesting that these hormones may exert some influence on Leydig cell function.

The administration of testosterone or estradiol decreased prostaglandin E_1 receptor concentration in rat Leydig cells. It is known that testosterone and estradiol have an inhibitory effect on the function of the rat testis. The inhibitory action of both these steroid hormones may be mediated by the hypothalamo-pitu-

itary system. A direct inhibitory effect of estradiol and testosterone on the function of the rat testis has been demonstated by Sairam and Berman (1979) and Purvis et al. (1979), respectively. Our results suggest that the effect of testosterone and estradiol on the function of the rat testis is a direct one, rather than mediated through the action of gonadotropins. PMSG and hCG have a stimulatory effect on Leydig cell function which is manifested by enhanced testosterone production. In our experiments the effect of both gonadotropins on PGE_1 receptors in rat Leydig cells was inhibitory. The inhibitory action of gonadotropins on PG receptors may be caused by increased secretion of testosterone. Cortisol was found to stimulate the formation of PGE_1 receptors, but ACTH which is known to stimulate the production of corticosterone as a main representative of corticoids in the rat failed to show such an effect.

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