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Testicular Adenyl Cyclase: Stimulation by the Pituitary Gonadotrophins

FREDERICK A. KUEHL, JR., DOLORES J. PATANELLI, JANET TARNOFF, AND JOHN L. HUMES

Merck Institute for Therapeutic Research, Rahway, New Jersey 07065

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Most peptide hormones including luteinizing hormone (LH) have been shown to effect their target organs by stimulating adenyl cyclase to produce adenosine 3′,5′-monophosphate, which acts as a second messenger to trigger the hormone-initiated events. Follicle stimulating hormone (FSH) has been an exception in that no effect of this hormone on the target adenyl cyclase has been reported in intact tissue. In the present studies, no effect of FSH could be detected on testicular slices of mature rats. However, when tissue from intact rats less than 21 days old or hypophysectomized rats was used, FSH was shown to markedly stimulate cyclic AMP production. Thus it is apparent that the FSH receptor site must be free from exposure to significant levels of endogenous hormone before an effect of exogenous FSH can be detected *in vitro*. Although LH is also capable of increasing cyclic AMP levels in testicular slices, this action occurs in tissue of both normal and hypophysectomized rats. Digestion of rat testes with collagenase permitted the preparation of substantial quantities of seminiferous tubule preparations, which were found to respond to FSH in a manner qualitatively similar to tissue slices. LH stimulated adenyl cyclase in isolated seminiferous tubules, and it is suggested that the action of LH in the testes is not restricted to the Leydig cells but that it also plays a role in conjunction with FSH in the growth of the epithelium of the seminiferous tubules.

Adenosine 3′,5′-monophosphate (cyclic AMP) has now been established as a second messenger mediating some of the effects of a number of hormones: vasopressin (Orloff and Handler, 1967), adrenocorticotrophin (Haynes *et al.*, 1960), thyroid-stimulating hormone (Gilman and Rall, 1966), and melanocyte-stimulating hormone (Bitensky and Burnstein, 1965). The action of luteinizing hormone (LH) has also been shown to be mediated through cyclic AMP in bovine corpus luteum tissue (Marsh *et al.*, 1966), and a similar sequence was suggested, but not established, in the male gonad by the observation that cyclic AMP increases testosterone production in rat testes (Sandler and Hall, 1966). In fact the manner in which FSH exerts its influence has received relatively little attention. Some workers found that it stimulates fluid imbibition and growth of the immature rat ovary (Steelman and Pohley, 1953; Evans and Simpson, 1950), and that gross changes induced in the ovary by FSH are accompanied by enhanced biosynthesis of protein and nucleic acid (Fiala *et al.*, 1957; Blackburn and Kostyo, 1965; Callentine *et al.*, 1965; Civen *et al.*, 1966). In *in vitro* studies with human ovarian slices, FSH has been reported to stimulate the conversion of testosterone to estradiol (Baggett *et al.*, 1956). In the intact immature male rat FSH was shown to stimulate growth of the testes (Greep *et al.*, 1942) and in the hypophysectomized adult rat to aid in the repair of the germinal epithelium (Lostroh, 1963) and affect morphological changes of the Sertoli cells (Murphy, 1965). Finally, although pretreatment of immature rats with FSH has been found (Means and Hall, 1967) to increase the ability of excised testicular tissue to incorporate labeled amino acids and to affect lipid

synthesis in the Sertoli cells (Lacy, 1967), the sequence of events prior to this and all the above mentioned effects has not been elucidated.

Subsequent to the completion of the present studies a stimulatory effect of LH and FSH on cyclic AMP formation in homogenates of dog testes was reported by Murad *et al.* (1969). Similar measurements with rat testes resulted in only minimal responses.

MATERIALS AND METHODS

Animals

Male CD rats, hypophysectomized at 21 days of age, were obtained from the Charles River Breeding Laboratories and fed *ad libitum* on a special diet containing 17% protein, fortified with vitamins A and D. Sexually mature rats of the ages indicated were obtained from the same source and fed Purina rat chow *ad libitum*. Weanling rats were produced in our own laboratories from Holtzman lineage.

Hormones

The bovine LH preparation used in these studies was NIH-LH-B5. Porcine FSH, PF-593-4-F (activity 3 × NIH-S-1), which contained less than 1% LH, was a gift from Dr. S. Steelman of these laboratories. The prostaglandins were gifts of Dr. Sune Bergström of Karolinska Institute, Stockholm, Sweden.

Experimental Procedures

Adenine-8-^{14}C, 50–55 mCi/mMole, was purchased from Schwartz BioResearch, Inc. Although it was 99+% pure, for the purposes of these studies it was necessary to purify it further. An aqueous solution (100 μCi/ml) of the nucleotide was adsorbed onto a bed of AG50W-X2[H+] 100–200 mesh, and the resin was washed first with 15 ml of water and then 17 ml of 0.1 M aqueous ammonia. The product was eluted with an additional 25 ml of 0.1 M aqueous ammonia, concentrated to dryness *in vacuo*, and dissolved in water to give a concentration of 100 μCi/ml.

Krebs-Ringer phosphate buffer with one-half the prescribed Ca++ concentration[1] and containing 6 mM glucose, pH 7.2, was used in these studies.

The rats were sacrificed by decapitation and the testes immediately excised. After the tunica albuginea was removed, the testes were placed on a filter paper moistened with isotonic saline maintained at 4C and sectioned with scissors into small segments. The resulting pieces were combined and randomized so that each incubate represented a minimum of four rats.

Suspensions of isolated seminiferous tubules were prepared by incubating 1–2g of testicular tissue freed of its tunica albuginea with 5 mg of bacterial collagenase [CLS-Worthington Biochemicals] in 5 ml of the Krebs-Ringer buffer at 32C for 3–5 min. Vigorous shaking was required during this incubation period. The resulting suspension was decanted onto a 4 × 4 inch piece of plastic screen, 1 mm^2, which rested on top of a petri dish. Separated tubules were immediately washed with 15 ml of buffer and transferred into a second petri dish containing 5 ml of buffer by inverting the screen into the buffer. The tubule suspension was then transferred to a 15-ml centrifuge cone and centrifuged for 3 min at 400g. After the buffer was decanted, the washing procedure was repeated two more times with additional 5-ml aliquots. The tubules were then transferred onto the surface of a pyrex spot testing plate, the indentations of which contained 0.5 ml of buffer. Equalization of the tubule conglomerates was accomplished by transfer from one well to another by means of forceps until approximately equal amounts of tissue were observed in each. Finally the tissue in each well was transferred with forceps into plastic incubation vessels containing 2 ml of buffer.

The effect of additions upon cyclic AMP levels was measured by the method described previously (Humes *et al.*, 1969a, b; Kuo and De Renzo, 1969). Pieces of intact tissue or portions of seminiferous tubules were placed in 2 ml of buffer containing 1 μCi of adenine-8-^{14}C (1×10^{-5}M) and incubated at 32C for 1 hr in a Dubnoff metabolic shaker set at 75 oscillations per minute. Theophylline (5×10^{-3}M) and gonadotropins were then added and the incubations continued an additional 30 min. The incubates were homogenized in the presence of 0.05 ml of a solution containing 10 μmoles/ml each of ATP, ADP, 5′-AMP, cyclic AMP, adenosine, and adenine to facilitate recovery of labeled nucleotides. Aliquots (0.05 ml) were withdrawn for protein determination, and the homogenates were immediately acidified with trichloroacetic acid to a final concentration of 2%. Cyclic AMP-^{14}C was isolated by the method previously described (Humes *et al.*, 1969) including paper chromatography. Protein determinations were performed by the Lowry method modified for use on the autoanalyzer.[2]

RESULTS

Initial studies employing slices of testicular tissue from mature rats revealed that maximal

[1] The medium had the following composition: NaCl 131 mM, KCl 5.2 mM, $CaCl_2$ 1.4 mM, $MgSO_4$ 1.3 mM, and Na_2HPO_4 10 mM.

[2] Lopez-Ramos, B., personal communication.

stimulation of cyclic AMP formation was achieved by 10 μg/ml of FSH and 0.1 μg/ml of LH. As shown in Fig. 1 essentially equal increments of cyclic AMP were obtained by these amounts of the two gonadotrophins; hence it is probable that the effect of FSH is attributable to contamination by the LH (ca. 1%) in this FSH preparation. However, when testicular tissue of 31-day-old rats hypophysectomized at 21 days of age was examined under the same conditions, the response to FSH but not LH was increased markedly. This increment cannot be attributed to LH contamination in the FSH preparation, since it represents a 2.5-fold excess in cyclic AMP production above that produced maximally by LH.

Digestion of testicular tissue, after removal of the tunica albuginea, with collagenase resulted in a rapid dissolution of connective tissue with the result that the seminiferous tubules could be separated easily from Leydig and other interstitial tissue cell types. Microscopic examination of such seminiferous tubules prepared from testes of 50-day-old rats revealed little contamination with adhering fragments of interstitial tissue, as shown in Fig. 2A and 2B. There are, however, some signs of rupturing of the seminiferous tubules with loss of Sertoli and germinal cells. Presumably this occurs as a result of handling this preparation. As indicated in Fig. 3 the effects of FSH and LH in increasing cyclic AMP levels in these isolated tubules prepared from testicular tissue of 28-day-old rats hypophysectomized at 21 days of age and 210-g intact rats approximately 50 days old are quantitatively similar to those elicited in the corresponding whole tissue.

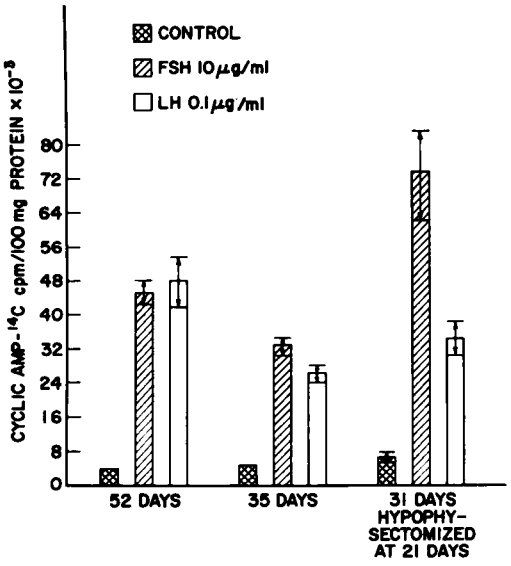

FIG. 1. The effect of FSH and LH on the stimulation of cyclic AMP synthesis in testicular tissue from adult, immature, and hypophysectomized rats. Pieces of testes, 60–100 mg wet weight, were incubated as described in Experimental Procedures. In this as well as in subsequent experiments all measurements were performed in duplicate.

The dose-response relationship of FSH and LH in testicular tissue of 31-day-old rats hypophysectomized at 21 days of age and tissue from 35-day-old intact rats in shown in Fig. 4. In the 0- to 1-μg/ml range of the gonadotrophins, it is clear (Fig. 4A) that testicular slices from intact rats are essentially nonresponsive to FSH compared to the same tissue of hypophysectomized rats in which the cyclic AMP levels rise dramatically with increased dosage. It can also be seen from these data (Fig. 4B) that the response curves obtained with LH in the two tissues closely follow each other until the 1-μg/ml level is approached, at which point the cyclic AMP produced is somewhat higher in tissue from hypophysectomized animals. The difference may be due to the contribution by the FSH in this LH preparation. As shown in Fig. 4C it is evident that a substantially higher quantity (1 μg/ml) of FSH is required to equal the cyclic AMP synthesis induced by 0.1 μg/ml of LH, which is the near maximal response level for this latter hormone in both tissues. Once the maximal response level of LH is reached in tissue from hypophysectomized rats, a sharp plateau is obtained whereas the stimulation by FSH continues to rise until at 5–10 μg/ml its ability to induce cyclic AMP formation exceeds that of LH by a factor of 2.5-fold on direct weight comparison. The possibility that FSH increases cyclic

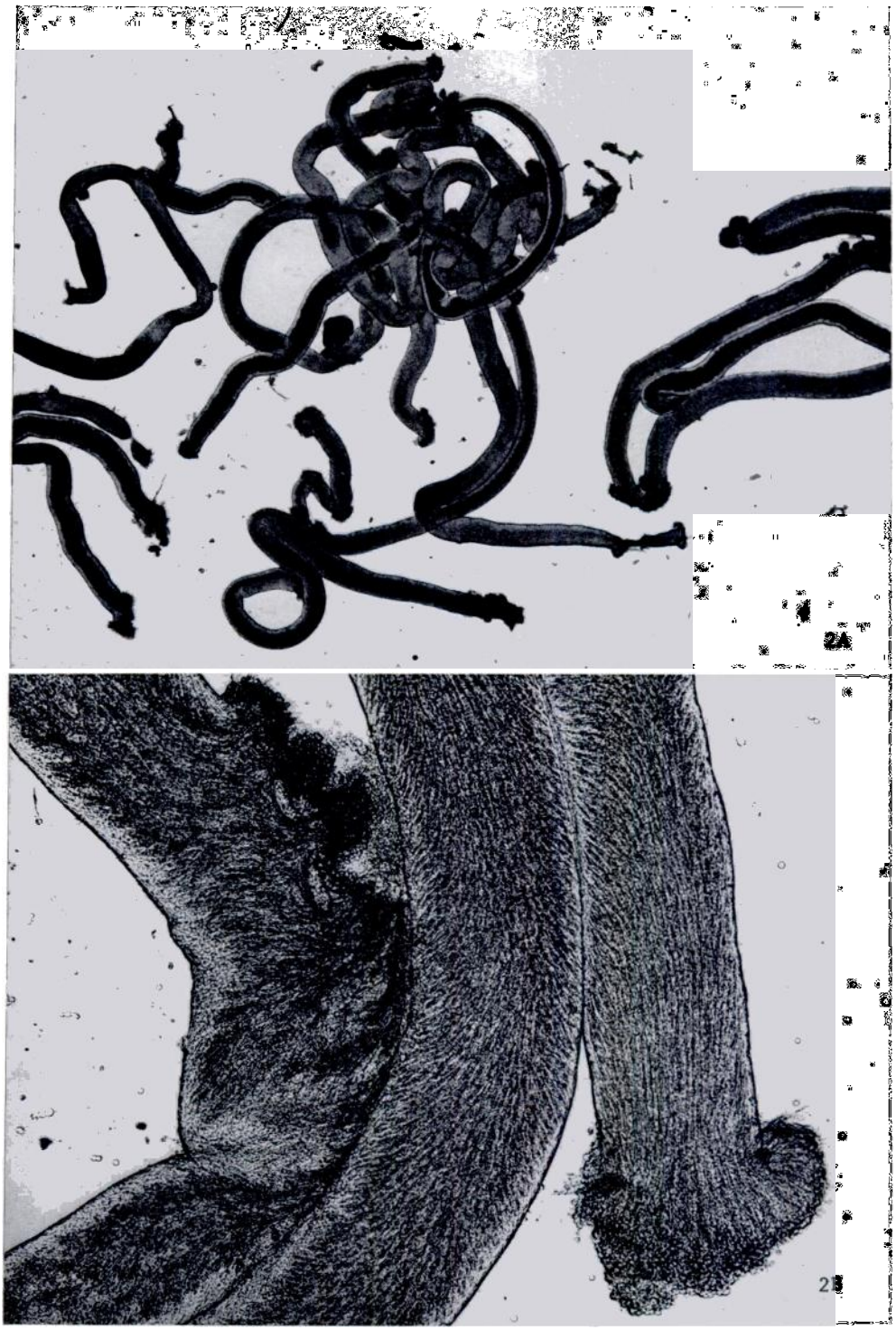

FIG. 2A. Photomicrographs of seminiferous tubules prepared from testicular tissue from 50-day-old rats. (unfixed ×10)

FIG. 2B. Isolated seminiferous tubules at higher magnification. (unfixed ×50)

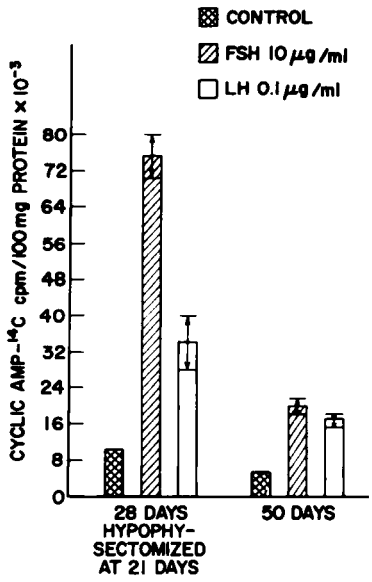

FIG. 3. The effect of FSH and LH on the stimulation of cyclic AMP synthesis in seminiferous tubules prepared from intact or hypophysectomized rats. Each incubate contained 6–9 mg protein as determined by the Lowry method.

AMP levels by inhibiting its conversion to 5′-AMP seems unlikely in view of the synergistic effect of theophylline upon its action. Direct evidence for this postulate is shown by the data in Table 1, where FSH is seen to have no effect upon phosphodiesterase activity in testicular tissue.

The effect of FSH and LH on cyclic AMP synthesis in testicular tissue of intact rats at various ages is presented in Fig. 5. Tissue from weanling rats is seen to respond to FSH in a manner qualitatively similar to testicular tissue or seminiferous tubules from hypophysectomized rats. The ability of FSH to elicit a "true cyclic AMP stimulation" (a stimulation of adenyl cyclase that cannot be attributable to LH contamination) diminishes with increasing age until it is lost by approximately Day 21.

The specificity of response of testicular tissue from 30-day-old rats hypophysectomized at Day 21 to the gonadotrophins is demonstrated in Table 2. The known adenyl cyclase

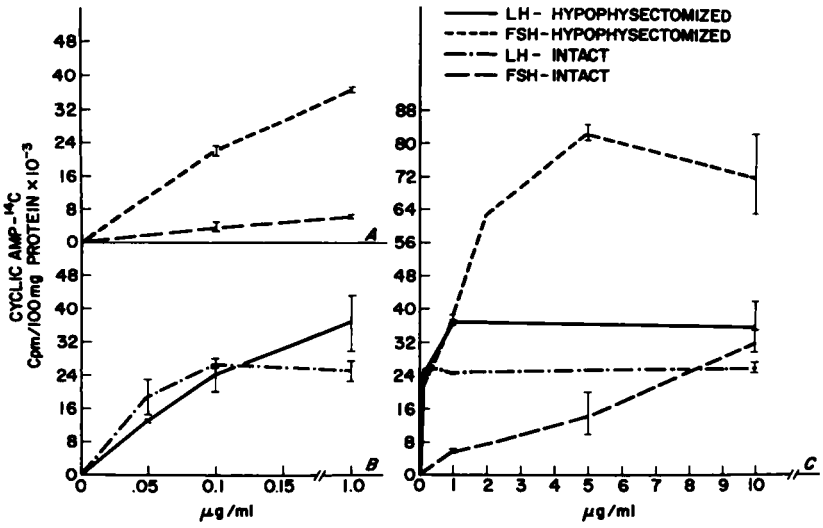

FIG. 4. The dose response relationship of FSH and LH on cyclic AMP synthesis in testicular tissue from 35-day-old intact rats and 31-day-old rats hypophysectomized at 21 days of age. Each incubate contained approximately 70–80 mg wet weight of tissue. Incubated control values of 9140 cpm/100 mg protein in the case of the intact group and 4190 cpm/100 mg protein for the hypophysectomized group were subtracted from all hormone treated incubates.

TABLE 1

EFFECT OF FSH, LH, AND THEOPHYLLINE ON CYCLIC AMP PHOSPHODIESTERASE ACTIVITY IN RAT TESTES

	Cyclic AMP hydrolyzed	
	mμMoles/mg protein/15 min	mμMoles/mg protein/30 min
Control	19.7	34.8
Theophylline 8.2 mM	3.5	5.9
FSH 10 μg/incubate	21.4	33.4
FSH 30 μg/incubate	21.4	36.0
LH 10 μg/incubate	16.0	28.0
LH 30μg/incubate	22.3	33.4

NOTE: Testicular tissue (4.3 g) from 30-day-old rats hypophysectomized at Day 21 was homogenized into 4.3 ml of 0.33 M sucrose at 4C. This homogenate was centrifuged at 2600 × g for 30 min at 4C. Aliquots of this supernatant containing 0.456 mg of protein were incubated at 31C for 15 or 30 min with 8.3×10^{-5}M cyclic AMP, 0.5 μCi cyclic AMP−^{3}H, 66 mM Tris•HCl, pH 7.5, 2mM $MgSO_4$ in total incubation volume of 0.3 ml. Aliquots, 30 μl, of the reaction along with unlabeled cyclic AMP, 5'-AMP, and adenosine were chromatographed on Whatman 3MM paper and developed for 16 hr in a solvent consisting of M ammonium acetate, pH 7.5, and ethanol (30:75). The nucleotides were visualized by ultraviolet light, and the areas of the chromatogram were cut out and placed in 20 ml of toluene scintillation fluid. In this system the predominant hydrolysis product was adenosine.

stimulators in other peripheral tissues (histamine, norepinephrine, isoproterenol, and ACTH) were less active than FSH at levels far higher than would ordinarily be employed with their appropriate target tissues. In addition prostaglandin E_1, prostaglandin $F_2\alpha$, and insulin, which have been shown to antagonize hormonally induced increases in cyclic AMP levels in fat cells, were without significant effect under the conditions studied. Growth hormone (NIH-GH-S-5) did cause a small enhancement in cyclic AMP synthesis; however, this response could be attributable to a small contamination by LH in the preparation.

DISCUSSION

LH has been shown to act in the ovaries to increase cyclic AMP levels (Marsh *et al.*, 1966), ultimately resulting in an increased production of progesterone. Using a radiochemical assay (Humes *et al.*, 1969) that measures hormonally stimulated cyclic AMP production, this observation has been confirmed with slices of bovine corpora lutea. Although cyclic AMP has been shown to increase testosterone synthesis in the male gonad, there has been no

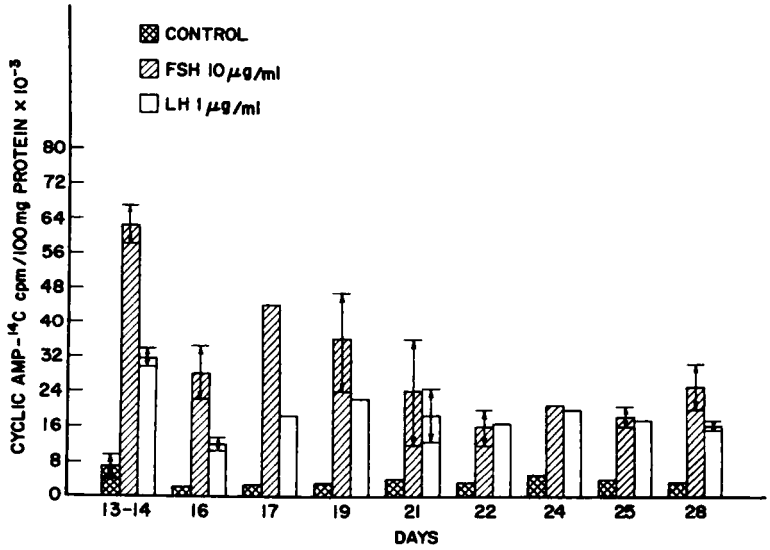

FIG. 5. The effect of FSH and LH on cyclic AMP synthesis in testicular tissue from weanling and immature rats.

TABLE 2

THE EFFECT OF KNOWN ADENYL CYCLASE STIMULATORS ON CYCLIC AMP SYNTHESIS IN TESTICULAR TISSUE FROM 30- OR 31-DAY OLD RATS HYPOPHYSECTOMIZED ON DAY 21

	Concentration	No. Obs.	cpm per incubate	Comments
Control		4	429 ± 37[a]	
Histamine	10 μg/ml	2	350–469	Inactive
Norepinephrine	10 μg/ml	2	713–1162	2.2X
Isoproterenol	10 μg/ml	2	706–601	1.5X
ACTH	10 μg/ml	2	515–838	1.6X
Growth hormone	10 μg/ml	2	1261–1426	3.5X
Insulin	1 mU/ml	1	462	Inactive
PGE_1	1 μg/ml	1	429	Inactive
$PGF_{2\alpha}$	0.5 μg/ml	1	535	Inactive
FSH	5 μg/ml	5	4534 ± 330[a]	10.6X
FSH + Insulin	5 μg/ml	2	4019–5775	No effect on FSH response
FSH + PGE_1	5 μg/ml	2	4924–6653	No effect on FSH response
FSH + $PGF_{2\alpha}$	5 μg/ml	2	4112–7385	No effect on FSH response
LH	1 μg/ml	2	2475–2659	5.9X
LH + Insulin	1 μg/ml	2	1544–2020	No effect on LH response
LH + PGE_1	1 μg/ml	2	1683–2251	No effect on LH response
LH + $PGF_{2\alpha}$	1 μg/ml	2	1524–2224	No effect on LH response

[a] Average ± SEM

NOTE: Two separate experiments were combined and the control and FSH values averaged. Approximately 100 mg of testicular tissue was present in each incubate.

direct evidence that LH stimulates adenyl cyclase in this organ. The present studies clearly demonstrate that LH does in fact stimulate adenyl cyclase in the testes as measured by the increment of newly formed nucleotide. It is evident that as in the ovaries the action of LH to induce steroid synthesis in the testes is mediated via cyclic AMP.

The present studies reveal that the effect of FSH in stimulating the conversion of ATP to cyclic AMP is of such a low order of magnitude in the testicular tissue of mature rats as to be accountable to trace contamination by LH. On the other hand, the response of the same tissue of immature male rats to FSH is of such a magnitude that it rules out an effect of LH contamination in the preparation and clearly indicates the presence of FSH-sensitive receptor sites. As shown in Fig. 5, the age at which the rats cease to respond to FSH is about Day 21. Histological studies of testes of rats 13 to 17 days of age revealed active proliferation of supporting cells, as well as spermatogonia. Maturation had proceeded to zygotene spermatocyte stage, and early pachytene spermatocytes were only seen occasionally. By the 18–19th day, the number of early spermatocytes was identical to that found in

the normal adult animal. It is interesting that at this time when cyclic AMP induced changes of the seminiferous epithelium would appear to be established (similar to those observed in the adult), the response to exogenous FSH ceases. It is also possible to demonstrate FSH stimulated cyclic AMP formation in testicular slices of 31-day-old rats hypophysectomized 10 days earlier (Fig. 1). This further strengthens the concept that the receptor site responsive to FSH is normally activated by endogenous hormone in maturing animals so that exogenous FSH is without effect. The fact that the basal rate of conversion of ATP to cyclic AMP is not substantially larger in testicular tissue of normal than in that of hypophysectomized rats remains unexplained (Fig. 1 and 3). Endogenous FSH would predictably be expected to enhance adenyl cyclase activity in the testes of the intact group. However, since the assay used in these studies measures only newly synthesized cyclic AMP as reflected by the measurement of labeled cyclic AMP, it is possible that higher levels of endogenous nucleotide present in testes of the intact group act either directly or indirectly to depress enzymatic activity by a feedback mechanism. In any event, a period free of exposure to endogenous FSH appears to be required before a stimulatory effect of added hormone can be noted *in vitro*. This obligatory requirement probably accounts for the absence of reports in the literature that the action of FSH, like that of LH and many other peptide hormones, is mediated through cyclic AMP.

Chase and Aurbach (1968) have shown that the rat kidney contains two adenyl cyclase receptor sites, one in the medulla responsive to vasopressin and the other in the cortex responsive to parathyroid hormone. The data presented herein suggest that a similar dual hormone adenyl cyclase receptor mechanism accounts for the action of the gonadotrophins in testes. It is not possible, however, to exclude the existence of a single receptor responsive to both LH and FSH on the basis of the present evidence. Nevertheless this seems unlikely in view of the altered response ratio to the gonadotrophins after hypophysectomy. This hypothesis is supported by the histological findings of Mancini *et al.* (1967), who report that fluorescence-labeled FSH and LH accumulate mainly in their target sites, the Sertoli and Leydig cells, respectively.

Seminiferous tubules of rat testes have been separated from interstitial tissue by microdissection (Christensen and Mason, 1965). This technique, although effective, is tedious and necessarily restricted to small scale preparations. Using a modification of the method used for the preparation of lipocytes (Rodbell, 1964) from rat epididymal fat pads, the present studies reveal that the connective tissue of the rat testes is particularly sensitive to the action of collagenase, leaving the seminiferous tubules relatively intact. This simple method is readily adaptable to large scale preparations. The viability of the seminiferous tubules so obtained is evident by the ability to incorporate uridine-^{14}C into RNA, leucine-^{14}C into protein, and glucose-1-^{14}C into $^{14}CO_2$.[3] Cellular integrity is further evident by the fact that activation of the adenyl cyclase by the gonadotrophins is unimpaired. As with tissue slices FSH is not effective on tubules of mature rats but very active in stimulating adenyl cyclase of the corresponding tissue of weanling and hypophysectomized animals. LH is about equally responsive in all of these groups. Surprisingly, the activation of adenyl cyclase in the tubules by FSH is not markedly greater than that of tissue slices, despite the greater concentration of Sertoli cells in these preparations. Also, the effect of LH is not in accord with the generally accepted hypothesis that the Leydig cell is the exclusive target for this hormone in the testes (Lacy, 1967). The concept that LH, in addition to its action on the Leydig cells, also plays an unique role in the development of the seminiferous tubules in conjunction with FSH is supported by bio-

[3] Unpublished observations.

logical data. Lostroh (1963) has reported that in hypophysectomized rats a small amount of LH is required along with higher doses of FSH to stimulate repair of the epithelium of the seminiferous tubules. Woods and Simpson (1961) have shown that this obligatory requirement of LH for full FSH activity can be duplicated by adding testosterone, the steroid whose formation is induced by LH. In studies in which the seminiferous tubules were mechanically separated from the interstitial tissue, Christensen and Mason (1965) found that both tissues have the capability of converting progesterone to testosterone, although the seminiferous tubules were the considerably less effective of the two in this regard. Thus, assuming a relationship between the levels of cyclic AMP produced and the subsequent biological events triggered, there is a reasonable quantitative relationship between the potencies of LH and FSH in stimulating their respective adenyl cyclases in the seminiferous tubules and their biological effects on the development of this tissue.

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