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FSH and LH/hCG Responsive Adenylyl Cyclases in Adult Rat Testes: Methodology and Assay Conditions

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The activities of the membrane bound testicular adenylyl cyclases (AC) and their responsiveness to LH/hCG and FSH were examined in membrane particles from adult rat testes. The assay requires the use of an ATP regenerating system consisting of creatine kinase, creatine phosphate, and myokinase for the maintenance of ATP levels. LH/hCG and FSH responsive AC in the rat testes showed the following properties:

1. GTP and GMP-P(NH)P showed a concentration-dependent stimulation of AC activity with an apparent K_m of $2 \times 10^{-6}M$.
2. The relative response to hCG and FSH was not influenced by ATP concentration if the free magnesium (Mg^{2+}) concentration was kept constant. The AC activity increased with increasing concentration of ATP (with an apparent K_m of approximately 1.0 mM) using Mg^{2+} of 0.4 mM in excess of ATP and EDTA.
3. At low ATP (0.15 mM) Mg^{2+} caused a concentration-dependent increase in both basal and hormone stimulated activities. At high ATP (1.65 mM) Mg^{2+} caused a concentration-dependent increase in basal AC activity, but a gradual decrease in hCG and FSH stimulated AC activities at Mg^{2+} concentrations higher than 5 mM.
4. Decreasing Mg^{2+} was associated with increasing relative responses to hCG and FSH. Optimal relative stimulation by FSH and hCG was obtained when Mg^{2+} was 0.4–1.0 mM in excess of the added Mg^{2+} binding ingredients (ATP and EDTA). Maximal relative stimulation by fluoride was achieved at much higher Mg^{2+} (5mM).

5. Increasing pH from 7.0 to 8.2 was associated with increasing basal activity. Optimal hormonal effects (relative response) were achieved using an incubation medium of pH of 7.00–7.40.
6. Time curves with GTP and GMP-P(NH)P with or without hormones showed linearity up to 20 min.
7. Hormonal activation by gonadotropins (hCG and FSH) was seen using both membrane particles and homogenates, and in the presence of either GTP or GMP-P(NH)P.
8. Relative responses to hCG and FSH were slightly better in membrane particles than in homogenates, and GMP-P(NH)P reinforced the FSH response better than GTP. Similar relative stimulation by hCG was obtained using both nucleotides.
9. Complete maintenance of AC activity as well as hormonal responses were seen after freezing at -70°C for two weeks.
10. LH/hCG responsive AC activity was associated with Leydig cells, whereas FSH responsive AC was entirely localized in the seminiferous tubules.

The finding of an FSH responsive AC in seminiferous tubules of adult rats does not support the notion that "uncoupling" of the FSH receptors from the AC is a primary reason for the loss of FSH response occurring during sexual maturation.

Key words: testis – adenylyl cyclase – FSH – LH/hCG.

The pituitary gonadotropins, LH and FSH, regulate testicular function via specific receptors localized on the Leydig cells and on the Sertoli cells, respectively (for reviews see Catt et al. 1974; Hansson et al. 1976; Means 1977). It is assumed that both these hormones regulate target cell function by modulating the activity of Leydig cell and Sertoli cell adenylyl cyclases (AC), which in turn stimulate the intracellular concentrations of cyclic AMP (cAMP).

In rats many of the rapid responses to FSH is lost during sexual maturation, and one explanation for this age dependent attenuation of the FSH response is an "uncoupling" of the FSH receptors from the AC (Means et al. 1978).

With few exceptions (Murad et al. 1969; Braun 1974; Braun & Sepsenwol 1976; Abou-Issa & Reichert 1979) knowledge of the role of the AC in gonadotropin activation of testicular target cells stems from measurements of cAMP released from living testicular cells after exposure to the tropic hormone (Means 1973; Braun & Sepsenwol 1974; Catt et al. 1974; Dorrington & Fritz 1974; Means et al. 1974; Mendelson et al. 1975), and an FSH responsive AC has not been directly demonstrated in postpubertal rats. We felt that a more detailed investigation of the regulation of FSH and LH/hCG responsive AC in a broken

cell system may give more direct information about the biochemical processes involved in gonadotropin activation of testicular target cells.

The data presently available indicate that the AC systems are complex enzymes regulated by guanylyl nucleotides, divalent cations, hormones and which use the MgATP complex as a substrate. Special attention was therefore given to the effects of these substances on the gonadotropin responsive AC in adult rat testes. The data indicate that adult rat testes contain both FSH and LH/hCG responsive AC which behave somewhat different from that reported for the gonadotropin responsive AC in ovarian tissues (Birnbaumer et al. 1976).

Materials and Methods

Animals

Adult male Sprague-Dawley rats were used. The animals were anaesthetized with ether and then sacrificed by cervical dislocation. Their testes were then removed and immediately placed on ice. The ages of the rats at the time of sacrifice are given in the figure legends.

Chemicals and enzymes

Creatine phosphate and creatine kinase were obtained from Calbiochem. [α - 32 P] ATP and [8- 3 H] cAMP were obtained from Amersham, England. Myokinase, Tris, ATP, cAMP, GTP, EDTA and sodium dodecylsulfate (SDS) were from Sigma Chemical Company. 5'-guanylylimidophosphate (GMP-P(NH)P) was obtained from Boehringer Mannheim.

Hormones

A commercial hCG preparation (Physex, LEO, Denmark) with an activity of 3000 IU/ampoule was used at a final concentration in the assays of 1 μ g/ml (10 IU/ml). Human FSH (hFSH-PT₁, 15 000 IU/mg by RIA) was a gift from P. Torjesen, Oslo. This hFSH preparation contained 1-2% hLH activity by RIA and was used at a final concentration of 1 μ g/ml. In some experiments a second hFSH preparation (hFSH-PT₂, 15 000 IU/mg by RIA) was used. It had an LH-contamination which was less than 0.4%. Ovine FSH (NIH-S12) was treated with antiserum against LH as described by Purvis et al. (1979). The hormone preparations were dissolved in distilled water containing 0.1% BSA (bovine serum albumin) prior to addition to the assays.

Preparation of homogenates

The testes were weighed, decapsulated and then homogenized in 20 volumes (with respect to the original tissue weight) of TE-buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.4) using an Ultra-Turrax homogenizer (Janke-Kunkel AG,

Germany) at rheostat setting 7 for 2×15 seconds. The homogenates were filtered through a nylon mesh and 20 μl of the homogenates were used for assay.

Preparation of membrane particles

If not specified otherwise the membrane particles were prepared as follows: Filtered homogenates were subjected to 2 centrifugations. The first centrifugation was carried out at $27\,000 \times g$ for 30 min at $0-4^\circ\text{C}$. The pellet was resuspended in 20 volumes (with respect to the initial tissue weight) of TE-buffer, rehomogenized using the Ultra-Turrax homogenizer for 10–15 seconds, and recentrifuged for 30 min at the same speed and temperature. The pellet was resuspended in 10 volumes of TE-buffer containing 0.1 % BSA using the Ultra-Turrax for 10–15 seconds.

Membrane particle suspensions and homogenates not to be used immediately were frozen at -70°C .

Dissection of seminiferous tubules (ST)

This was carried out in Eagles medium at room temperature using two pairs of tweezers. The testes was teased apart, and ST were dissected using the method of Christensen and Mason (1965). The isolated tubules were collected and weighed. The membrane particle suspensions were then prepared as described above.

Adenylyl cyclase (AC) assay

AC activity was determined in 20 μl aliquots of homogenates or membrane particles diluted in homogenization medium (25–95 μg protein per assay tube) in a final volume of 50 μl containing 0.1–2.0 mM ATP ($2-18 \times 10^6$ cpm of [α - ^{32}P]ATP), 1.0–30.0 mM MgCl_2 , 1 mM EDTA, 1 mM cAMP (with ca. 10 000 cpm [^3H]cAMP), 20 mM creatine phosphate, 0.2 mg/ml creatine kinase, 0.02 mg/ml myokinase, and 25 mM Tris-HCl buffer, pH 7.4. Incubations were carried out at 30°C or 35°C for 2–20 min. The reactions were stopped with 0.1 ml of a "stopping solution" containing 10 mM cAMP, 40 mM ATP, and 1 % sodium dodecylsulfate (SDS) followed by mixing and immediate cooling to 0°C . If time curves of cAMP accumulation were to be determined, the final reaction volumes were approximately 1.1 ml, and the reaction was stopped at the indicated times by removal of 50 μl aliquots which were immediately added to 0.1 ml "stopping solution" followed by mixing and cooling to 0°C .

The [^{32}P]cAMP formed and [^3H]cAMP added to monitor recovery were then isolated according to the method of Salomon et al. (1974) using Dowex chromatography (Krishna et al. 1968) and alumina chromatography (Ramachandran 1971; White & Zenser 1971) with minor modifications described by

Birnbaumer et al. (1976). Overall recovery was 65–75 %, and reaction blanks ranged from 3–6 cpm per 1×10^6 cpm of labelled ATP added. The imidazol eluates from the alumina columns were collected in scintillation vials containing 5 ml Insta gel (Packard). Radioactivity was measured in a Nuclear Chicago, Mark I liquid scintillation counter.

The efficiency of the regenerating system was monitored by subjecting an aliquot of the incubated and stopped reaction mixture to thin layer chromatography on plastic-backed polyethyleneimine (PEI) cellulose plates (Brinkman) using 1 M Tris-HCl as the developing medium. After chromatography the per cent of added [α - 32 P]ATP remaining as such was determined by liquid scintillation counting.

Protein was determined by the method of Lowry et al. (1951), using BSA as the standard.

The ATP, GTP and GMP-P(NH)P concentrations used were calculated by optical density measurements at 260 nm.

Results

Activation of FSH responsive testicular adenylyl cyclase (AC) by GTP and GMP-P(NH)P

Since most hormone responsive AC systems are influenced by guanylyl nucleotides, we examined the effect of varying concentrations of GTP and GMP-P(NH)P on FSH responsive testicular AC in the presence of saturating concentrations of FSH. As seen from Fig. 1, both GTP and GMP-P(NH)P caused a concentration-dependent stimulation of AC activity both with an apparent K_m of approximately 2×10^{-6} M. GMP-P(NH)P plus hFSH-PT₂ induced AC activation (approximately 11.5 times the basal value) and so did GTP (approximately 3.1 times). In these studies it was important to use reagents free of "GTP-like" contaminants. An ATP preparation synthesized from adenosine (Sigma cat. No. A 2383) and a purified regenerating system prepared as described by Iyengar et al. (1979) were found adequate. On the basis of these findings we decided in subsequent studies to use GTP or GMP-P(NH)P concentrations of 10–20 $\times K_m$ ($2\text{--}4 \times 10^{-5}$ M) in order to saturate the guanylyl nucleotide binding sites on the hormone receptor adenylyl cyclase complex.

Effects of ATP concentration

Previous studies by Birnbaumer et al. (1976) have shown that the LH responsive AC in the corpus luteum is dramatically influenced by the concentration of ATP. Not only was an increasing basal and hormone stimulated

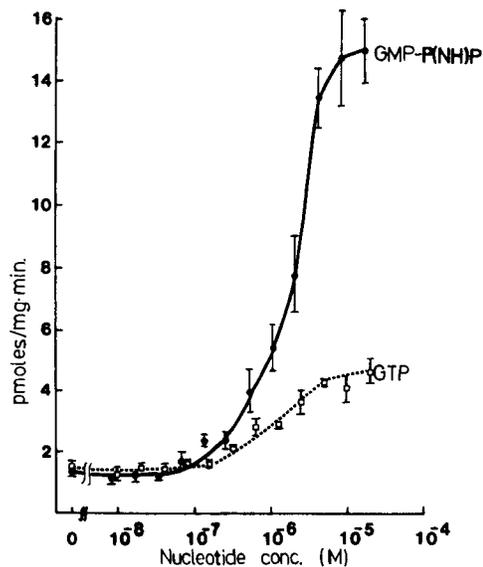


Fig. 1.

Adenylyl cyclase (AC) activity in terms of cAMP formed per mg protein per min in rat testes as a function of GTP and GMP-P(NH)P concentrations. AC assay was as described in Materials and Methods. Other conditions for assay were: ATP 0.91 mM and Mg^{2+} 3.5 mM. Membrane particles (90.4 μ g protein per assay) from 16 month old rat testis were used. A part of the testis was homogenized in 20 volumes of TE-buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.4), filtered, and centrifuged at $27\,000 \times g$ for 30 min at 0–4°C. The final pellet was resuspended in 5 volumes of TE-buffer containing 0.1% BSA. Incubations were carried out at 30°C for 10 min in the presence of 1.8 μ g/ml hFSH-PT₂. A purified regenerating system free of GTP was used. Each point represents a mean \pm SD (standard deviation) of triplicate incubations.

activity with increasing concentrations of ATP seen, but the relative response to LH was increased from 2-fold to 9-fold when ATP concentrations were increased from 0.1 to 10 mM. For that reason we examined the effect of increasing concentrations of ATP on basal, LH/hCG and FSH stimulated AC activities and determined to what extent the relative stimulation of testicular AC activities by hormones was affected by ATP concentrations. In view of the dramatic effects of magnesium (Mg^{2+}) (see later), and the fact that ATP binds Mg^{2+} , it was essential to keep free Mg^{2+} concentrations constant in excess of the Mg^{2+} binding ingredients (ATP and EDTA) in the medium. As shown in Fig. 2, increasing concentrations of ATP at a constant excess of Mg^{2+} of 0.4 mM showed a concentration-dependent increase in both basal and hormone stimulated AC activities, however, the relative responses both to hCG and FSH showed very little changes.

Effects of Mg^{2+}

The dependence of AC systems and their stimulation by hormones on Mg^{2+} is complex. For that reason we investigated the effect of varying concentrations of Mg^{2+} on basal and hormone stimulated AC activities at two different ATP concentrations in the presence of saturating concentrations of either GTP or GMP-P(NH)P. As seen in Fig. 3, Mg^{2+} caused a concentration-dependent stimulation of both basal as well as hormone stimulated AC activities. When GMP-P(NH)P was used, basal, LH/hCG and FSH responsive AC activities were higher than when GTP was used. However, while relative stimulation by hCG was of the same magnitude regardless of nucleotide, FSH stimulated

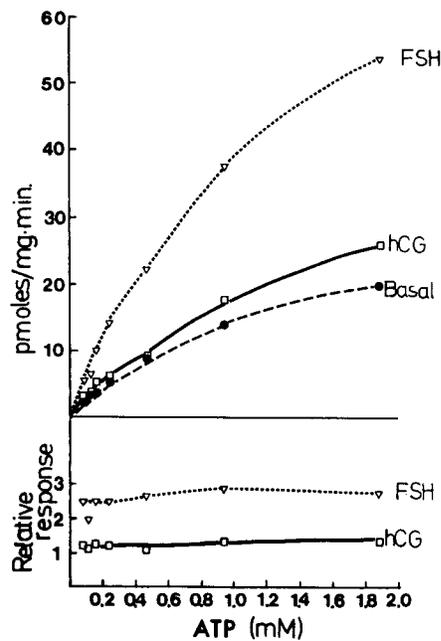


Fig. 2.

Effect of ATP on activity and hormonal responsiveness of rat testicular AC. Activities were determined in the presence of 0.04 mM GMP-P(NH)P. The upper ordinate shows cAMP formed per mg protein per min. Membrane particles (49.5 μ g protein per assay) from the testis of a 74 days old rat were used. Incubations were carried out at 35°C for 15 min under standard assay conditions described under Materials and Methods. The hormones had the following final concentrations: hCG (1 μ g/ml), hFSH-PT₂ (2 μ g/ml). Each point represents the mean of triplicates, and the standard deviation (SD) was never more than 10 per cent of the plotted values. Relative response means the absolute activity of the stimulated responses divided by basal activity.

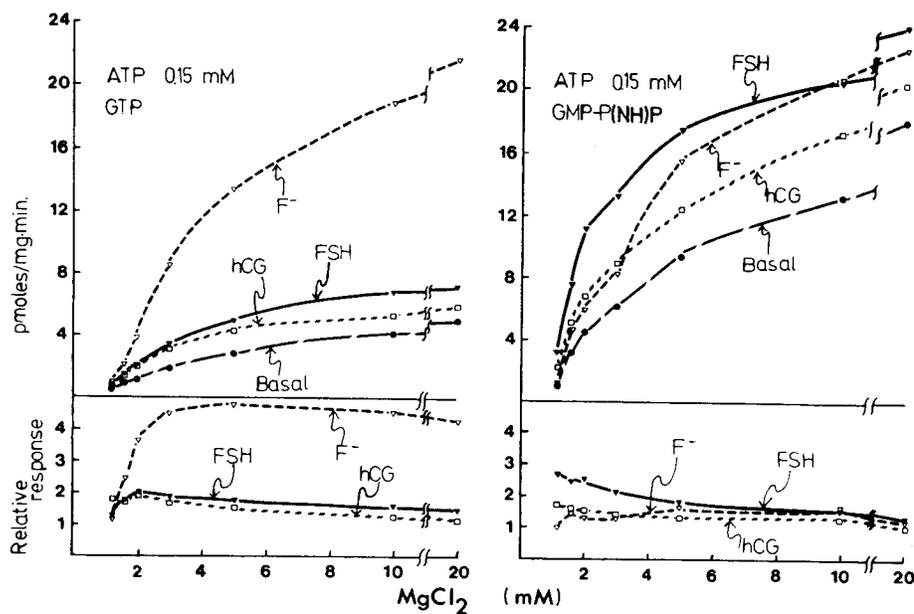


Fig. 3.

Effect of the Mg^{2+} concentration at low ATP (0.15 mM) on activity and hormonal responsiveness of the AC in rat testes. Activities cAMP formed per mg protein per min were determined in the presence of GTP 0.04 mM (left panels) and GMP-P(NH)P 0.04 mM (right panels). The upper panels show the absolute activities, the lower panels the relative activities (hormone stimulated activities relative to basal activities). Left panels: Membrane particles (65.6 μ g protein per assay) from 73 days old rat testis were used. The testis was decapsulated and a part of the testis was then homogenized in 10 volumes of TE-buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.4), filtered, and centrifuged at $270 \times g$ for 10 min at $0-4^{\circ}C$. This was carried out to remove tissue fragments, nuclei, and major cell debris. The supernatant was centrifuged at $27\,000 \times g$ for 30 min at $0-4^{\circ}C$. The final pellet was resuspended in 5 volumes of TE-buffer containing 0.1% BSA. Right panels: Membrane particles (83.2 μ g protein per assay) from 72 days old rat testis were used. The procedure for making membrane particles is the same as mentioned above. Incubations were carried out at $35^{\circ}C$ for 10 min under standard assay conditions described under Materials and Methods. Other conditions for assay were: hCG 1 μ g/ml, hFSH-PT₂ 2 μ g/ml, fluoride (F^{-}) 10 mM. Each point represents the mean of triplicate incubations, and the standard deviation (SD) was never more than 5 per cent of the plotted values. See also Fig. 4.

AC activity was constantly better using GMP-P(NH)P than using GTP. (See also Table 1).

Fluoride stimulated AC activity on the other hand was not increased by substituting GMP-P(NH)P for GTP; this resulted in a decreased relative response to fluoride ion in the presence of the analogue. An important feature

Table 1. LH/hCG and FSH responsive AC in homogenates or membrane particles using either GTP or GMP-P(NH)P. The homogenate and membrane particles were prepared from 90 days old rat testes as described in the legend to Fig. 9, and had been frozen for 13 days prior to the study. AC activities in terms of cAMP formed per mg protein per min were determined in the presence of either GMP-P(NH)P 0.04 mM or GTP 0.04 mM. Incubations were carried out at 35°C for 10 min. Conditions for assay were: ATP 0.94 mM, Mg²⁺ 2.8 mM, hCG 1 μg/ml and hFSH-PT₂ 2 μg/ml. Values are mean ± SD of triplicate determinations. Levels of significance were determined by Student's *t*-test.

		GTP			GMP-P(NH)P		
		Basal	hCG	FSH	Basal	hCG	FSH
Homogenate	AC activity	2.0 ± 0.3	3.3 ± 0.2*	5.2 ± 0.1**	3.7 ± 0.2	6.0 ± 0.1**	10.9 ± 0.2**
	Rel. response		1.6	2.6		1.6	2.9
Membrane particles	AC activity	2.8 ± 0.3	5.1 ± 0.4*	8.1 ± 0.2**	8.8 ± 0.6	14.1 ± 0.2**	31 ± 3**
	Rel. response		1.8	2.9		1.6	3.5

* $P < 0.005$

** $P < 0.001$

that emerged from this experiment (Fig. 3) and which can be seen more clearly in Fig. 4 is that the greatest relative hormonal responses were obtained at the lower Mg^{2+} concentrations. However, the fluoride response showed a concentration-dependent stimulation by Mg^{2+} with a maximum of about 5–10 mM.

To test the efficiency of the regenerating system, the incubation mixtures of the experiment shown in Fig. 3 were examined by thin layer chromatography using PEI cellulose plates. There appeared to be an increasing efficiency of the regenerating system with increasing Mg^{2+} , yet in all cases ATP levels after incubation, even at low ATP, was greater than 60 % of the starting level (not shown).

Effects of pH

The LH responsive AC in the ovary shows a pH optimum between 7 and 8. We examined in detail, basal, LH/hCG, FSH and fluoride stimulated AC activities in the range of pH 7.0 to 8.2, both at high and low ATP. In both cases free Mg^{2+} was 0.4 mM in excess of Mg^{2+} binding ingredients in the incubation. As seen in Fig. 5, there was a tendency towards an increase in both basal and stimulated activities with increasing pH, particularly at low ATP concentrations. However, maximal relative response were seen at pH 7.0 to 7.4 with a gradual decrease thereafter. Under these assay conditions (low Mg^{2+} and using GMP-P(NH)P as the guanylyl nucleotide) the fluoride response was low and showed, if anything, a small decrease with increasing pH. A pH in the physiological range appears to be adequate for studying hormonal activation of AC in the testes of adult rats.

Time course and AC activity

The results depicted in Fig. 6 show the time course of basal, LH/hCG and FSH stimulated AC activity in membrane particles from adult rat testes in the presence of either GTP (left) or GMP-P(NH)P (right). We wanted to examine whether or not the rate of cAMP formation was constant during a time period of 20 min using the optimal conditions described above (Mg^{2+} 0.4 mM in excess of ATP and EDTA, pH 7.4, and GTP and GMP-P(NH)P 0.04 mM). Fig. 6 shows that this is the case. In the case of GTP, the time course curves of both basal and hormone stimulated AC activities appear to intercept in the origin. In the case of GMP-P(NH)P, basal and hormone stimulated AC activities were linear after a delay of approximately 2 min. The addition of hormones (hCG or FSH) appeared to reduce this delay to some extent but did not eliminate it. Thus, from these experiments we concluded that assay of AC activities between 10 and 15 min would give a meaningful estimate of AC activity.

Fig. 7 shows results from experiments in which varying amounts of enzyme protein was added. AC activities were determined in the presence of 0.04 mM

GMP-P(NH)P and saturating concentrations of hCG. As seen from the figure there was a linear increase in AC activity between 14 and 77 μg of protein per tube.

Preparation and storage of enzyme

Fig. 8 shows basal activity and hormonal activation using either homogenates or testicular membrane particles. Basal activity and the effects of saturating concentrations of hCG or FSH were compared in homogenates, in resuspended testis membrane particles washed once (membrane particles I) or twice (membrane particles II) with 20 volumes of TE-buffer. As seen from the figure, there was no difference in hCG response regardless of enzyme preparation used.

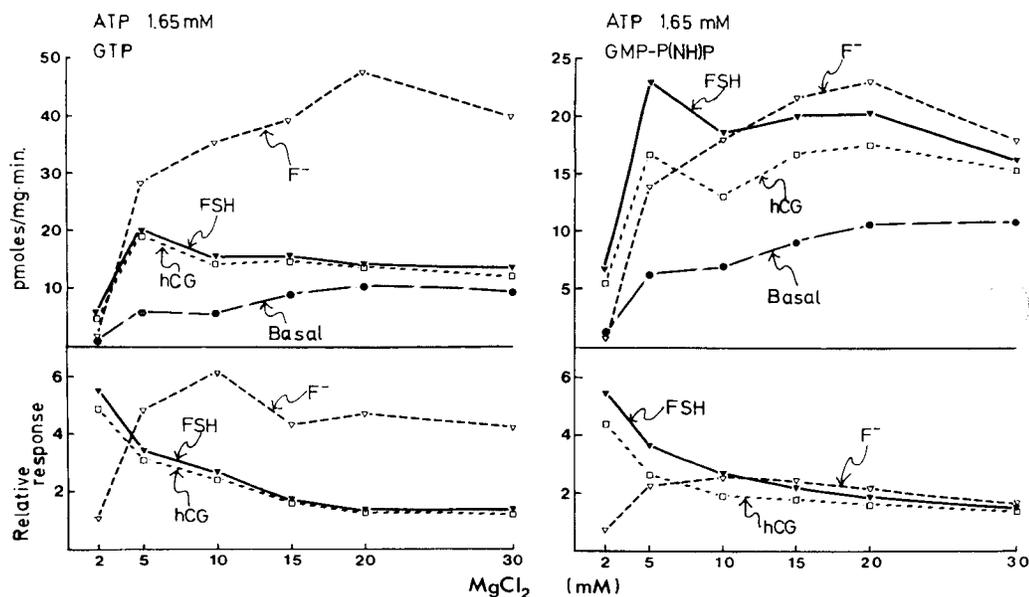


Fig. 4.

Effect of Mg^{2+} concentration at high ATP (1.65 mM) on activity and hormonal responsiveness of the AC in adult (70 days old) rat testes. Activities (cAMP formed per mg protein per min) were determined in the presence of GTP 0.04 mM (left panels) and GMP-P(NH)P 0.04 mM (right panels). The upper panels show the absolute activities, the lower panels the relative activities (hormone stimulated activities relative to basal activities). Membrane particles (28 μg protein per assay) from intact adult rats were used. Incubations were carried out at 30°C for 10 min under standard assay conditions described under Materials and Methods. Note the different scales at the ordinates. Each point represents the mean of triplicate determinations, and the standard deviation (sd) was never more than 10 per cent of the plotted value. See also Fig. 3. \square — \square : hCG (1 $\mu\text{g}/\text{ml}$), \blacktriangledown — \blacktriangledown : hFSH-PT₁ (1 $\mu\text{g}/\text{ml}$), ∇ — ∇ : F⁻ (fluoride) (10 mM).

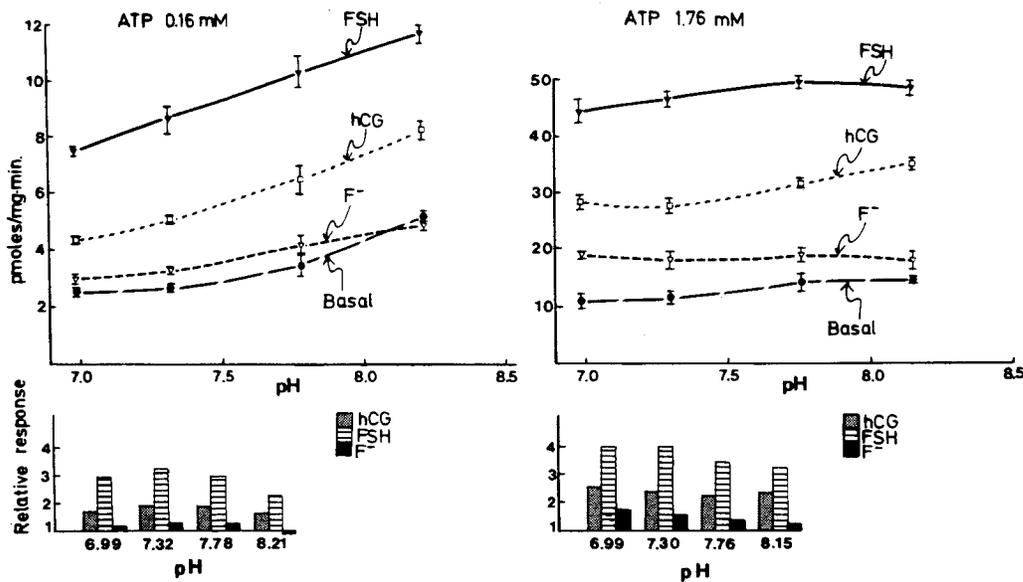


Fig. 5.

Effect of the pH on basal, LH/hCG and F⁻ stimulated AC activities in the presence of GMP-P(NH)P 0.04 mM and Mg²⁺ 0.4 mM in excess of ATP and EDTA. Membrane particles from 65 days old rat testis were used. The pH values reported are those determined in the incubation medium before incubation. The corresponding pH values under incubation were 7.10–7.35–7.65–8.00. Incubation were carried out at 35°C for 12 min. The relative activities were calculated by dividing stimulated activities by the corresponding basal activities. The rest of the incubation conditions are described in the figure and in Materials and Methods. The following final hormone concentrations were used: hCG (1 μg/ml), hFSH-PT₁ (1 μg/ml). Note the different scales at the ordinates.

Each point represents the mean ± SD of triplicate determinations.

Left panel: Low ATP (0.16 mM). Right panel: High ATP (1.76 mM).

However, the FSH response was consistently better using membrane particles than homogenates.

Fig. 9 shows the stability of AC activity in homogenates or membrane particles assayed immediately, or after storage at -70°C for 2 days or 13 days, respectively. The assays were carried out on 3 different occasions. As seen from the figure, there was no change in relative response either to hCG or to FSH. The minor changes in absolute AC activities may be due to minute differences in Mg²⁺ and ATP concentrations between the three different assays.

As seen in Fig. 3, GMP-P(NH)P seemed to support the FSH response better than GTP, whereas no difference was seen with regard to the hCG response. The data shown in Table 1 show the effect of hCG and FSH on AC

activities in homogenates or membrane particles using either GTP or GMP-P(NH)P as the nucleotide. In both homogenate and membrane particles GMP-P(NH)P gave greater basal and hormone stimulated activities, and a better relative FSH response than when using GTP. Again the response to FSH was better in membrane particles than in homogenates.

Specificity and compartmentalisation of the FSH response

Since the FSH preparations were contaminated with small amounts of LH (Fig. 10), we tested the specificity of the FSH response in two ways: First we absorbed an ovine FSH preparation with a highly specific anti-LH antiserum, after which all the Leydig cell stimulating activity was lost (Fig. 10). Furthermore, we demonstrated that the various FSH preparations stimulated AC activity in isolated seminiferous tubules, in which no LH/hCG responsive AC activity was present (Fig. 11). As seen from Fig. 11, the human FSH pre-

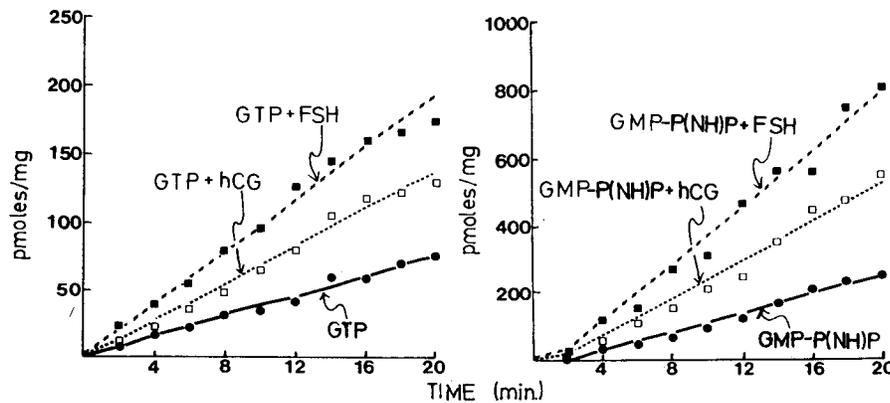


Fig. 6.

Time course of AC activity in testis particles from a 65 days old male rat. The ordinates show cAMP formed per mg protein. Membrane particles (70.4 μ g protein per assay) were used. Testis tissue was homogenized in 20 volumes of TE-buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.4), filtered, and centrifuged at $27\,000 \times g$ for 30 min at 0-4°C. The pellet was resuspended in the same volume of TE-buffer, rehomogenized and recentrifuged at the same speed and temperature. The final pellet was resuspended in 10 volumes of TE-buffer containing 0.1% BSA. Incubations were carried out at 35°C. Conditions for assay: ATP 1.7 mM, Mg^{2+} 0.4 mM in excess of ATP and EDTA, hCG (1 μ g/ml), ovine FSH (NIH-S12) with antiserum against LH (13.5 μ g/ml). Each point represents single determination.

Left panel: Time course of AC activity in testis particles using 0.04 mM GTP.

Right panel: Time course of AC activity in testis particles using 0.04 mM GMP-P(NH)P.

Note the different scales at the ordinates.

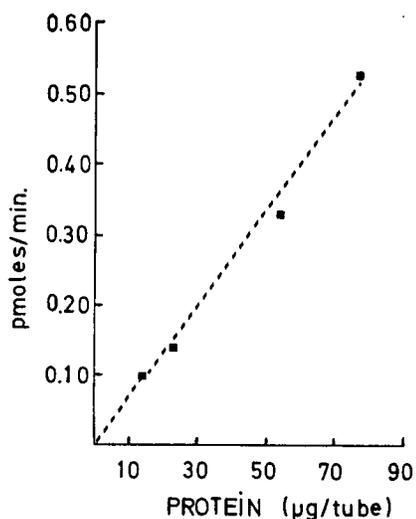


Fig. 7.

Protein dose response curve. Activities (pmoles cAMP formed per min) were determined in the presence of GMP-P(NH)P 0.04 mM and hCG 1 µg/ml. Membrane particles were prepared from 100 days old rat testis as described in Materials and Methods. The final pellet was resuspended and diluted in 4 different volumes of TE-buffer containing 0.1 % BSA. Incubations were carried out at 35°C for 12 min. Conditions for assay were: ATP 1.01 mM, Mg²⁺ 2.8 mM. Each value represents the mean of triplicate determinations, and the standard deviation (SD) was never more than 10 per cent of the plotted value.

parations were 10 to 16 times more potent than the absorbed ovine FSH (NIH-S12) (ED₅₀ for hFSH-PT₂, hFSH-PT₁, and ovine FSH was 83, 140 and 1320 ng/ml, respectively). Furthermore, ovine FSH absorbed with the anti-LH antiserum did not stimulate the AC activity in isolated Leydig cells (not shown).

Discussion

In this study we are attempting to present some guidelines for the assay of LH/hCG, FSH and fluoride responsive AC activity in testicular tissue from adult rats, especially with respect to assay conditions and the preparation of the suspension containing membrane bound AC. The present study is the first demonstration of an FSH responsive AC in seminiferous tubules of adult rats. It confirms the specific role of guanylyl nucleotides in the regulation of AC activity in the rat testes (Abou-Issa & Reichert 1979). Abou-Issa & Reichert found that GMP-P(NH)P plus FSH and GTP plus FSH caused an augmentation

of AC activity in immature rat testes of 6 times and 1.4 times above the basal level, respectively. In our study guanylyl nucleotides gave a much higher stimulation of AC activities (11.5 and 3.1 times, respectively) in adult rat testes. The much more dramatic effect seen in our study can be due to several factors:

1) We investigated testes from adult rats, whereas Abou-Issa & Reichert investigated testes from immature rats.

2) In our studies we used ATP preparations and a purified regenerating system both of which were essentially free of GTP. In order to see a GTP

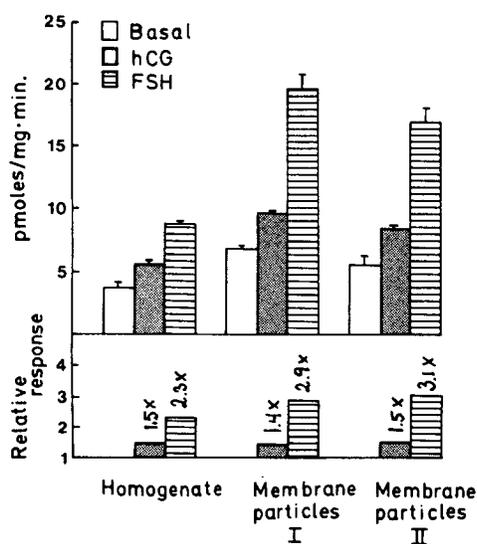


Fig. 8.

Basal AC activity and hormonal activation using homogenate or membrane particles from adult (65 days old) rat testis. Homogenate (77.6 μg protein per assay) was prepared as described in Materials and Methods. Membrane particles were prepared in two ways:

I. Testis tissue was homogenized in 20 volumes of TE-buffer, filtered, centrifuged at $27\,000 \times g$ for 30 min at $0-4^\circ\text{C}$ and resuspended in 10 volumes of TE-buffer + 0.1 % BSA. Twenty μl (53.2 μg protein per assay) of the membrane particle suspension were added to each assay tube.

II. These were prepared as above, except they were washed once more in 20 volumes of TE-buffer, recentrifuged at $27\,000 \times g$ for 30 min at $0-4^\circ\text{C}$ and finally resuspended in 10 volumes of TE-buffer containing 0.1 % BSA. Twenty μl (44.1 μg of protein per assay) of this membrane particle suspension were added to each assay tube. Incubation were carried out at 35°C for 10 min. Conditions for assay were: ATP 0.94 mM, GMP-P(NH)P 0.04 mM, Mg^{2+} 2.8 mM, hCG 1 $\mu\text{g}/\text{ml}$, hFSH-PT₂ 2 $\mu\text{g}/\text{ml}$. The bars show mean values \pm SD of triplicate determinations.

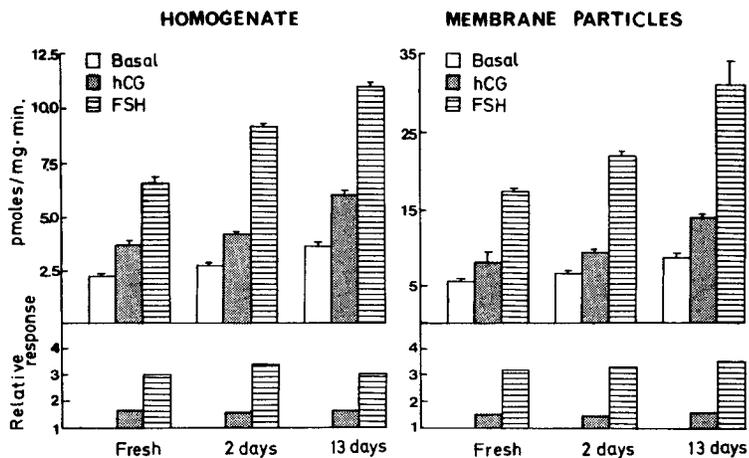


Fig. 9.

The stability of homogenates and membrane particles to storage at -70°C . Activities, cAMP formed per mg protein per min, were determined in the presence of GMP-P(NH)P (0.04 mM). Homogenate (left panel) and membrane particles (right panel) from 90 days old rat testes were prepared as described in Materials and Methods. The homogenate and membrane particles were used for the determination of AC activity either on the same day (fresh) or after 2 and 13 days of storage at -70°C . Incubations were carried out at 35°C for 10 min. Conditions for assay were: ATP 1.00 mM (fresh), 1.01 mM (2 days), 0.94 mM (13 days), Mg^{2+} 2.8 mM, hCG 1 $\mu\text{g}/\text{ml}$, hFSH-PT₂ 2 $\mu\text{g}/\text{ml}$. Note the different scales at the ordinates.

The bars show mean values \pm sd of triplicate determinations.

stimulation of AC, an ATP preparation and a regenerating system free of GTP will lower the basal activity and thus amplify the relative response. Furthermore, it has been shown that GTP competitively inhibits the enzyme activation caused by GMP-P(NH)P (Lefkowitz 1974; Salomon et al. 1975; Abou-Issa & Reichert 1979).

3) In our studies we used a lower free Mg^{2+} concentration than that used by Abou-Issa & Reichert, which also appears to give a higher relative response (Figs. 3 and 4).

It has been suggested (Rodbell et al. 1971; Lad et al. 1977), that in addition to a nucleotide binding site on the AC, there is also a nucleotide binding site on the hormone receptor which may be important for the hormonal activation of the AC. In most systems GTP seems to be the preferred nucleotide. However, in the rabbit corpus luteum system it has been suggested that ATP is the nucleotide important for "receptor activation" and coupling of the hormone receptor complex to the AC (Birnbaumer & Yang 1974). We therefore examined

the influence of varying ATP concentrations on AC activity. In so doing we took into account the Mg^{2+} binding properties of ATP, and kept the free Mg^{2+} concentrations constant (0.4 mM in excess of ATP and EDTA). It was of interest to explore whether a similar "coupling" role could be ascribed to ATP in the stimulation of testicular AC. As shown in Fig. 2, ATP concentration did not affect responsiveness to hCG. Thus it appears that the coupling mechanism intervening between LH receptor and testis (Leydig cell) AC differs in its nucleotide dependency from that intervening between LH receptor and corpus luteum AC.

The Mg^{2+} effects on testicular AC (Figs. 3 and 4) are significant, and both at low and high ATP concentrations the free Mg^{2+} should be kept at a very low level (around 0.4 mM) in order to get an optimal hormonal response. However, under these circumstances absolute activities are very low. In certain instances, in which total activities are low, it may be necessary to increase the Mg^{2+} concentrations in order to stimulate the total AC activity to within the measurable range.

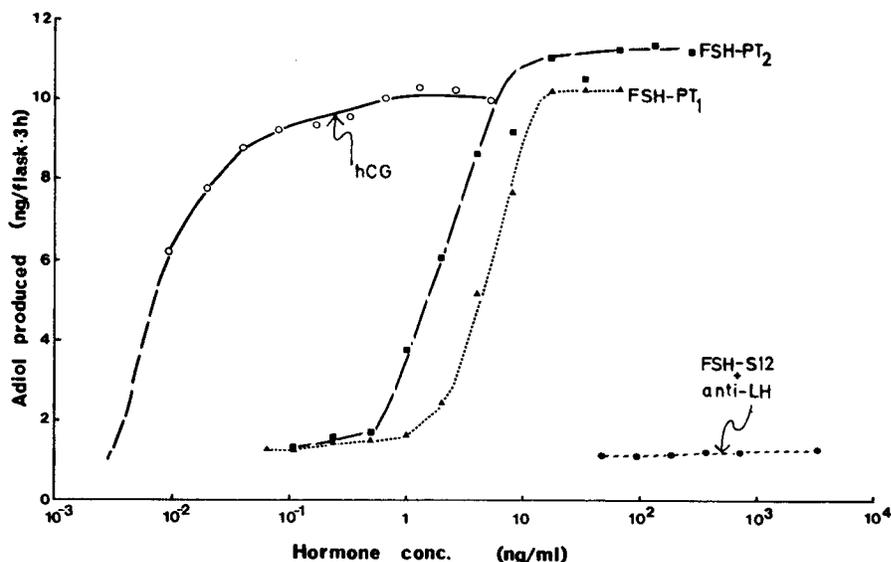


Fig. 10.

Comparison of the Leydig cell stimulating activity of 4 hormone preparations: human chorionic gonadotropin, hCG (○—○), two human FSH preparations, FSH-PT₁ (▲—▲) and FSH-PT₂ (■—■) and an ovine FSH (NIH-S12) preparation preabsorbed with LH antiserum, FSH-S12 + anti-LH (●—●). Leydig cells were prepared from immature (25 day) rats and the response was evaluated in term of 5α -androstane- $3\alpha,17\beta$ -diol (adiol) secretion. Each point is the mean of duplicate measurements.

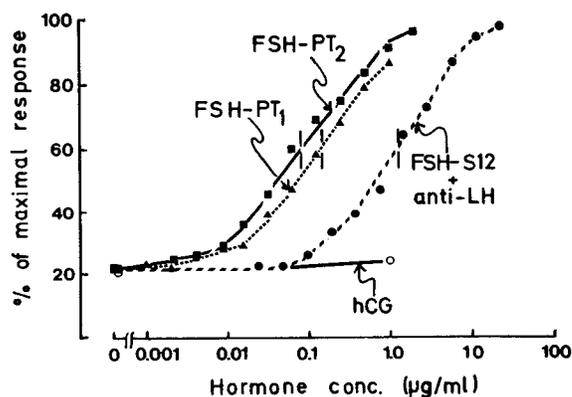


Fig. 11.

Concentration response curves for different FSH preparations. Seminiferous tubules were dissected from three 90 days old normal rats, and were homogenized in 20 volumes of TE-buffer, filtered, and centrifuged at $27\,000 \times g$ for 30 min at $0-4^{\circ}\text{C}$. The pellet was then resuspended in 10 volumes of TE-buffer containing 0.1% BSA. The incubations were carried out at 35°C for 10 min. Conditions for assay were: ATP 1.87 mM, GMP-P(NH)P 0.04 mM, Mg^{2+} 3.5 mM. FSH-PT₁ and FSH-PT₂ are human FSH preparations. FSH-S12 + anti-LH is an ovine FSH preparation (NIH-S12) treated with antiserum against LH. Basal AC activity (without FSH) was 5.6 pmoles/mg prot. \times min and maximal AC activity (with FSH) was 26.6 pmoles/mg prot. \times min. Each point is the mean of duplicate determinations.

Increasing the pH in the physiological range (between 7.0 to 8.2) caused a slight increase in both basal and hormonal stimulated activities. The highest relative responses were seen between 7.0–7.4. At this point it is not clear how changing pH causes alterations in AC activity, although it is possible that the effect may be mediated through the degree of dissociation of MgATP complex to free Mg^{2+} , ATP^{3-} and ATP^{4-} (De Haën 1975; Rendell et al. 1975; Salomon et al. 1975; Birnbaumer et al. 1976).

From a practical point of view it is noteworthy that GMP-P(NH)P seems to support the FSH response better than the LH/hCG response. Thus, in order to obtain a maximal relative response to FSH, GMP-P(NH)P is the preferred nucleotide. Since the guanylyl nucleotide binding site on the AC complex may be a GTP-ase, it may be that in the adult rat there is in the Sertoli cell a higher GTP-ase activity than in the Leydig cell. Thus, the distinct difference between GTP and GMP-P(NH)P in relation to the FSH response may be physiologically relevant.

The relative FSH response was usually greater in isolated seminiferous tubules (4 to 6 fold) than in whole testes, probably due to a decreased basal activity

after elimination of the interstitial tissue. Furthermore, the dissection time in tissue culture medium at room temperature (approximately 1 h) needed to isolate the seminiferous tubules may cause a considerable dissociation of endogenously bound FSH to the tubules and thereby also decrease basal activity and increase the relative response.

Van Sickle & Means (1979) reported that adult rat testes may contain a soluble "uncoupling" factor which may explain the lack of FSH response in the postpubertal testes. The present demonstration of an FSH responsive AC both in homogenates and washed membrane particles in the presence of either GTP or GMP-P(NH)P does not support this hypothesis.

The finding of a slightly reduced relative response in homogenates compared to the membrane particles from the same homogenates is probably due to a decrease in basal AC activity rather than to an increase of hormone stimulated AC.

Recent studies examining FSH binding and FSH responsive AC through sexual maturation (days 9-71) show an excellent correlation between FSH receptors and FSH responsive AC during this time period (Jahnsen & Hansson, in preparation).

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