REGULATION OF TESTICULAR LH RECEPTORS BY HOMOLOGOUS HORMONE: IN VITRO STUDIES ON RECEPTOR OCCUPANCY AND RECEPTOR LOSS

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SUMMARY

A method is described which makes use of 4M $MgCl_2$ to dissociate the testicular luteinizing hormone-receptor complex without altering either the binding capacity or binding affinity of the receptor. Using this method, it was demonstrated that in vitro incubation at 4° of decapsulated rat testes with various concentrations of luteinizing hormone or with human chorionic gonadotropin resulted in a reduction in binding capacity. This reduction of binding capacity could not be completely accounted for by occupation of receptors by homologous hormone, suggesting that receptors were lost. Thus negative regulation of LH receptors by LH and hCG was observed. The reduction in LH binding capacity was specific for LH and hCG, dose dependent and time related. FSH, prolactin and growth hormone did not exert the same effect.

LH* or hCG* initiates its action in the testis by binding to specific membrane receptors of Leydig cells. Although it has been reported that less than 1 per cent occupation of these receptors is necessary to elicit a maximum response in testosterone production (testicular responsiveness), it is not known whether the non-occupied receptors are really "spare" and nonessential. We recently demonstrated that hypophysectomy of adult male rats leads to an 85% reduction of available testicular LH receptor sites and a 60% reduction in testicular responsiveness six days following surgery (1). If LH administration is initiated at the time of hypophysectomy and continued twice daily for six days, testicular responsiveness is maintained at normal or above normal levels, while available LH receptor sites are even lower than in the hypophysectomized saline injected control animals (1). These studies suggest that maximal responsiveness can be elicited with relatively few receptors. To determine whether the apparent low levels of

*Abbreviations: Luteinizing hormone, LH; human chorionic gonadotropin, hCG; follicle stimulating hormone, FSH.

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available receptor sites are due to occupancy or due to actual loss of receptors, a method for dissociation of LH from its receptor is essential. The present report describes such a method which results in the dissociation of bound hormone from testicular receptor preparations without altering the binding capacity or affinity of these receptor preparations.

MATERIALS AND METHODS

Mature male rats, 75-85 days old, were obtained from Holtzman Co., Madison, Wisc. Ovine LH (NIH-LH-S19), ovine FSH (NIH-FSH-S11), ovine prolactin (NIH-P-S12) and ovine growth hormone (NIH-GH-S11) were obtained from NIH, National pituitary agency. HCG (10,000 units USP/mg) used for *in vitro* incubation was obtained from Ayerst Co. Rats were killed by decapitation, testes were quickly removed, decapsulated and incubated at 4° in 2 ml of 0.25M sucrose containing 10 mM Hepes buffer pH 7.4, 0.1% BSA and 5 mM CaCl (buffer A), and various concentrations of ^{125}I-hCG, LH, FSH, prolactin or growth hormone as indicated in text. At the appropriate time, incubations were stopped by transferring the testis into a beaker containing 50 ml ice-cold buffer A and washing briefly. For the 0 time points, ice cold decapsulated testis were put into hormone containing buffer for less than 30 seconds and immediately transferred to 50 ml of cold buffer. Each testis was then cut in half and each half processed separately as described below. One half of each testis was homogenized very gently in 1 or 2 ml of 4M $MgCl_2$ solution, being very careful to avoid any foaming. Immediately following homogenization the $MgCl_2$ homogenate was diluted with 15 volumes of buffer A. The other half of each testis was homogenized in 1 or 2 ml buffer A and diluted 15-fold with the same buffer. The homogenates were centrifuged at 20,000xg for 30 min. The 20,000xg pellets were washed in 6 ml of buffer and centrifuged again at 20,000xg for 30 min. These pellets were resuspended and aliquots used to measure LH receptor concentration.

The LH receptor assay was performed in 0.2 ml final volume by incubating 100 λ of the resuspended pellet with a saturating concentration of ^{125}I-hCG in buffer A in 12x75 mm disposable polypropylene tubes previously coated with 5% bovine serum albumin. Nonspecific binding was determined in the presence of the same amount of ^{125}I-hCG and tissue preparation plus 500-fold excess of unlabeled hCG. The reaction mixtures were incubated for 90 min at 34°(maximal binding was achieved by 60 min and did not change up to 180 min). Reactions were stopped by addition of 1 ml of ice-cold buffer and centrifugation at 20,000xg for 30 min. The resulting pellet was washed two times with 1 ml buffer A. Bound radioactivity was determined by a Packard gamma spectrometer. Specific hormone binding was calculated as the difference between binding in the presence and absence of excess unlabeled hormone and expressed as pmol per testis on the basis of the measured specific activities and active fractions of the ^{125}I-hCG preparation.

RESULTS

To test whether treatment with 4M $MgCl_2$ was effective in removal of bound hCG, decapsulated testes were incubated with ^{125}I-hCG (300,000-1,000,000 cpm). At the end of the incubation period, testes were removed

Table 1. Effect of 4M $MgCl_2$ homogenization on ^{125}I-hCG binding to 20,000xg pellet*

Experiment	N	Buffer Homogenization Homogenate (cpm)	20,000xg pellet (cpm)	Per Cent Bound	$MgCl_2$ Homogenization Homogenate (cpm)	20,000xg pellet (cpm)	Per Cent Bound
I	(4)	9711±681	9486±384	98±1.3	7133±176	877±163	12.5±2.5
II	(5)	66817±709	54064±432	81±0.5	50861±2424	4797±282	0.6±0.2
III	(4)	6237±45	5874±133	94±2.7	5904±233	750±30	13.0±0.82

*Data represents mean ±S.E.

Table 2. Effect of $MgCl_2$ treatment on ^{125}I-hCG binding capacity/testis.*

Experiment	N	Buffer (pmol ^{125}I-hCG bound/testis)	$MgCl_2$ (pmol ^{125}I-hCG bound/testis)
I	(2)	3.40±0.04	3.62±0.09
II	(10)	3.56±0.08	3.36±0.19
III	(6)	3.32±0.04	3.40±0.21

*Data represents mean ±S.E.

from the media and washed to remove unbound ^{125}I-hCG. Testes were cut in half, one half testis was homogenized with 4 M $MgCl_2$ and the other half testis was homogenized in buffer. Each testis homogenate was divided into 4 or 5 equal aliquots and counted prior to dilution with buffer and centrifugation as described in Methodology. Table 1 illustrates that between 81 and 98% of the radioactive counts which were associated with the testis are specifically bound to the 20,000xg testicular fraction. However if the testis was homogenized in 4 M $MgCl_2$, only approximately 10% of the total counts were associated with the pellet. These results suggest that 4 M $MgCl_2$ dissociated 90% of the bound hormone from receptor.

To determine whether $MgCl_2$ treatment damages LH receptor sites, testes were cut in half, one half of each testis was homogenized in buffer and the other half in 4 M $MgCl_2$ and the 20,000xg pellets were collected as described under Methods. The 20,000xg pellets were resuspended and the binding capacity of the preparation was measured. The results as presented in Table 2 demonstrate that $MgCl_2$ treatment does not damage the binding capacity of the

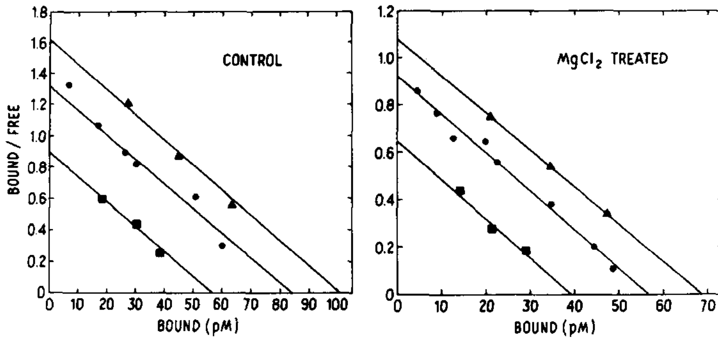

Fig. 1. Scatchard analysis of binding kinetics for ^{125}I-hCG to suspensions of testicular 20,000g pellet. A. Testis homogenized in buffered sucrose. B. Testis homogenized in 4 M $MgCl_2$. Receptor preparations were diluted 1/8 (▲), 1/10 (●), 1/15 (■).

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testis. To demonstrate that $MgCl_2$ homogenization has no effect on binding affinity Scatchard analysis of ^{125}I-hCG binding was carried out on control and $MgCl_2$ treated 20,000xg pellet. Fig 1 represents the results from experiments utilizing three different dilutions of receptor preparations. As can be seen there is no difference between the K_a of control and $MgCl_2$ treated preparations, $K_a = 1.60 \times 10^{10}$L/mol and 1.59×10^{10}L/mol respectively. These results indicate that $MgCl_2$ treatment does not alter either the binding capacity or the binding affinity of the testicular preparations for ^{125}I-hCG.

Having established that $MgCl_2$ treatment could be used to dissociate LH/hCG from its receptor without damaging receptors, this method was then utilized to determine the relationship between LH binding and the disappearance of LH receptors. Decapsulated testes were incubated in buffer containing 0.17 μM, 0.25 μM or 2.5 μM LH at 4° for various periods of time. As is illustrated in Fig 2, solid line, there is a marked reduction in hCG binding capacity per testis. The degree in reduction of hCG binding and the time interval necessary to reach minimal binding capacity is dependent on the concentration of LH. The higher the concentration of LH in the medium, the more rapid is the rate of decrease in binding, and the greater the reduction in

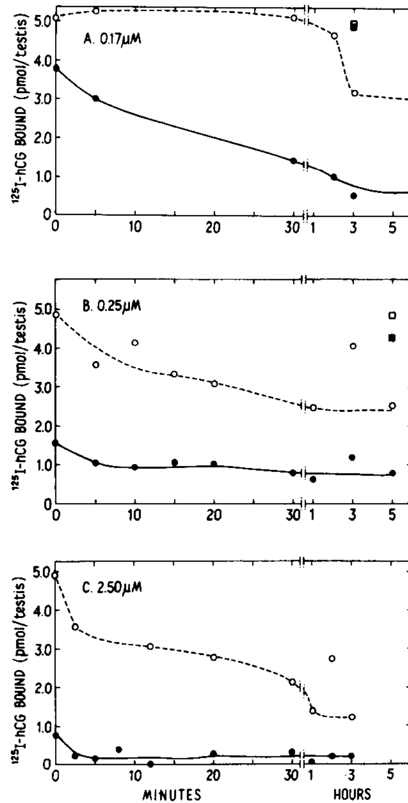

Fig. 2. Effect of LH (NIH-LH-S19) on binding capacity of testis in vitro. A. 0.17 μM (10 μg/ml). B. 0.25 μM (15 μg/ml). C. 2.5 μM (150 μg/ml). (—●—) Buffer control, (--o--) $MgCl_2$ treated, (■) Control testis (no LH), buffer control, (□) Control testis (no LH)$MgCl_2$ treated.

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binding capacity. The time interval required to reach maximal reduction in total binding was ∿ 30, 5 and 2.5 min for 0.17, 0.25 and 2.5 μM LH, respectively. To establish how much of this decrease in binding capacity was a result of occupation by LH and how much was due to disappearance of the receptor, ^{125}I-hCG binding capacity after removal of bound LH was determined in half of each testis after $MgCl_2$ treatment (Fig 2, broken line). At 0 time, ^{125}I-hCG binding capacity after $MgCl_2$ treatment was identical to values obtained from testes incubated in the absence of LH (Fig 1a,1b(■), homogenized in buffer; (□), homogenized in 4 M $MgCl_2$). However, at later time intervals,

Table 3. Effect of various peptide hormones on ^{125}I-hCG binding capacity.*

Treatment 100 μg/ml	Buffer (pmol ^{125}I-hCG binding capacity/testis)	$MgCl_2$
None	4.10±0.850	3.90±0.280
LH	0.06±0.028	0.45±0.055
hCG	0.79±0.290	2.02±0.350
FSH	3.32±0.480	3.35±0.380
Prolactin	3.17±0.520	3.27±0.460
Growth Hormone	2.28±0.230	2.88±0.220

*Data represents mean ±S.E.

a dose dependent decrease in hCG binding capacity was observed which was not a result of occupation. The decrease in binding capacity measured after treatment with $MgCl_2$ was from ∿ 5 pmol/testis observed in control testes to ∿ 3, 2 and 1 pmol/testis for 0.17 μM, 0.25 μM and 2.5 μM LH* respectively. The time required to reach maximal disappearance of LH receptor sites also appeared to be dose dependent, 3 h for 0.17 μM LH and 1 h for 0.25 and 2.5 μM LH. The difference in hCG binding capacity between $MgCl_2$ treated testes and buffer treated controls (broken line vs solid line, Fig 2) is considered to represent occupation of LH receptors by added LH and the decrease in hCG binding capacity observed in $MgCl_2$ treated testes is considered to represent actual disappearance of LH receptor sites.

To determine whether the observed decrease in LH/hCG receptor sites is specific for LH/hCG, testes were incubated for 3 h with 100 μg/ml of the following hormones: LH, hCG, FSH, prolactin and growth hormone. The results are presented in Table 3. LH and hCG resulted in both occupation and disappearance of LH receptor sites while incubation in the presence of FSH and prolactin had no effect on either occupation or disappearance of LH receptor sites. Incubation in the presence of growth hormone resulted in some occupation and also some disappearance of LH receptor sites. This can be attributed to the LH contamination of the growth hormone preparation which is equivalent

*Concentration of LH was calculated on a basis of NIH-LH-S19 being 50% pure LH and a molecular weight of 30,000 dalton.

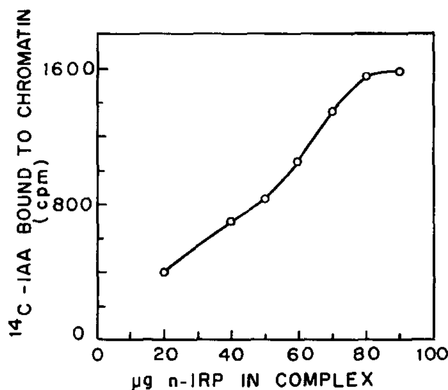

Fig. 3. Saturation of chromatin binding of [^{14}C]IAA-n-IRP complex. Increasing amounts of the complex, isolated by DCC treatment were added to a fixed amount of chromatin with DNA equivalent 42 μg. The complex, prepared by incubating 1 ml n-IRP (200 μg/ml) in 10 mM Tris-Cl$^-$, pH 8.0 with 1.8 x 10^{-6}M [^{14}C]IAA at 25° for 30 min., then adding 1 ml DCC suspension shaking for 30 min. and centrifuging, contained 17,500 cpm [^{14}C]IAA/100 μg IRP/ml. Different aliquots of the complex were incubated with the chromatin solution in 10 mM Tris-Cl$^-$ buffer, pH 8.0 in a total volume 1 ml at 0° for 30 min. and washed with the same buffer on Millipore filters. The filters were dried and counted in toluene based liquifluor.

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synthesis has been noted though it cannot be said with certainty what type of RNA synthesis is stimulated in this case (Table 2). It is evident that a non-responsive chromatin like that of chicken erythrocyte is not affected by the complex. A stimulation in the heterodispersed RNA was however noted when the product labelled RNA was electrophoresed in 3% polyacrylamide gel (Fig. 4). This is also observed when homologous RNA polymerase (nonribosomal) from the coconut chromatin was used. It thus appears that the *E. coli* RNA polymerase can transcribe this chromatin with some fidelity as has been reported with other eukaryotic systems *in vitro* (21). The stimulation was found to be significant in 9 - 12S RNA. Similar observation

decreases in ^{125}I-hCG binding to testicular preparations following *in vivo* administration of LH or hCG. Because of an observed difference in rates of decrease in hCG binding with two different doses of hCG, these authors suggested that negative regulation of gonadotropin receptors occurs. However, their studies did not distinguish between occupancy and loss of receptors.

Mechanism involved in the loss of receptors are still unknown. Our observation that disappearance of LH receptor sites occurs very rapidly and at 4° *in vitro*, makes it unlikely that the mechanism involved in negative regulation of receptor sites requires protein synthesis. The data suggest that the loss of receptor sites is a result of hormone-receptor interaction in the membrane and probably does not involve intracellular processes. The fate of the lost receptor remains to be ascertained.

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