INTRACELLULAR UPTAKE AND CATABOLISM OF LUTROPIN BY TESTICULAR TISSUE IN VIVO

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Received 19 January 1977

1. Introduction

The mechanisms involved in the expression of biological activity of lutropin have been extensively studied [1] and, analogous to other peptide hormones, the initial step is believed to involve binding of the hormone to a specific receptor located in the plasma membrane of the target cells. The specificity and characteristics of hormone binding to testicular [1] and ovarian [2] receptors have been investigated and preparations of partially purified receptor are available [2–4]. Little is known, however, about the fate of the hormone–receptor complex and its relationship, if any, to the termination of biological activity. Interestingly, it has recently been reported that the number of rat testicular gonadotropin receptors decreased with time following injection of either lutropin or choriogonadotropin [5].

Studies in this laboratory have been concerned with the kinetics and mechanisms of plasma clearance and biotransformations of lutropin [6–10]. The results of in vivo studies on the kinetics of lutropin uptake and catabolism by target tissue are presented in the present communication. As expected, tritium labeled ovine lutropin binds specifically to the testes following intravenous injection into mature male rats. From sucrose gradient centrifugation, it appears that at least some of the hormones which are bound to Leydig cells are internalized and eventually degraded by lysosomes. This phenomenon suggests a possible mechanism for terminating hormone action by target cells and may be important in the regulation of lutropin receptors by the gonadotropin itself.

2. Materials and methods

Ovine pituitary lutropin, with a potency of 1.97 × NIH-LH-S18 (95% confidence limits = 1.27–3.53), was a highly purified product prepared and assayed as described elsewhere [7,11]. Tritium labeling by reductive methylation to give a highly active product containing [^{3}H]monomethyllysine and [^{3}H]dimethyllysine was conducted using the method given earlier [12]. After gel-filtration, the [*methyl*-^{3}H]lutropin used in these studies had a specific radioactivity of 20.8 Ci/mmol and a potency of 1.93 × unlabeled lutropin (95% confidence limits = 1.76–2.12), i.e., 3.8 U/mg.

Total acid phosphatase (EC 3.1.3.2) activity was measured in 0.1% (w/v) Triton X-100 using *p*-nitrophenylphosphate (Sigma) as substrate and monitoring the absorbance at 405 nm for *p*-nitrophenol production. The reaction conditions and reagents represented slight modifications [13] of existing methods [14,15]. Free acid phosphatase activity was measured by a standard assay [16,17]. The activity of 5′-nucleotidase (EC 3.1.3.5) was determined as the difference in the amount of inorganic phosphate released from sodium 5′-AMP (Sigma) in the absence and presence of 25 μM α,β-methylene-5′-ADP (P-L Biochemicals). The reaction was carried out as described elsewhere [13] and terminated by the addition of 10% (w/v) TCA. The samples were centrifuged and the inorganic phosphate released was measured in the supernatant [18]. α,β-Methylene-5′-ADP is a competitive inhibitor of 5′-nucleotidase [19] and its incorporation into the enzymic assay permits the specific measurement of the

enzyme in the presence of non-specific phosphatases [20]. Protein was measured by the method of Lowry et al. [21] using bovine serum albumin as standard.

Mature male rats were injected intravenously [7], generally with 50 μg [*methyl*-^{3}H]lutropin, and sacrificed at the times indicated. The testes were removed, decapsulated, placed on ice, and washed with cold 0.25 M sucrose, 10 mM Tris–HCl, pH 7.4. Homogenization was carried out in the same medium using 50 strokes of the tight pestle of a Dounce homogenizer. Initially, subcellular fractions were prepared by the method of Mego and McQueen [22].

Sucrose-gradients were prepared at room temperature using a Beckman Density Gradient Former and were stored at 4°C for 18 h prior to use. The testicular particulate fraction (27 000 × *g*, 15 min) 0.5 ml in Tris-buffered sucrose, was carefully layered on 12.5 ml gradients and centrifuged at 35 000 rev./min for 2 h in a Beckman L2-65B ultracentrifuge at 4°C with a SW41 rotor. Thirteen fractions (1.0 ml each) were collected from the top using a Buchler Autodensiflow and analyzed for radioactivity and enzymic activity. Refractive index measurements on control gradients without tissue established fraction densities.

Discontinuous sucrose gradient centrifugation was performed using a Beckman 60 Ti rotor at 31 000 rev./min for 60 min at 4°C. The testicular particulate fraction was resuspended by homogenization in 20 ml 59.9% sucrose (ρ = 1.22 g/ml). Thirteen ml of this suspension were placed in a 30 ml centrifuge tube and overlayered with 10 ml 48.45% sucrose (ρ = 1.18 g/ml), followed by a 4 ml layer of 42.9% sucrose (ρ = 1.16 g/ml). All sucrose solutions contained 1 mM $CaCl_2$ and %-solution refers to w/v. Fractions of 2 ml were collected.

Total and TCA-soluble radioactivity were determined as described elsewhere [7]. TCA-soluble products were prepared using techniques given elsewhere [13] and the separation of amino acids from oligopeptides was accomplished using copper–Sephadex chromatography [23].

Disruption of subcellular fractions by Triton X-100 and osmotic shock were based on standard methods [14,24]. Disruption by homogenization was accomplished using a Polytron PT-10 homogenizer at 15 000 rev./min for 5 min. Afterwards, the particles were centrifuged at 27 000 × *g* for 20 min and the supernatant was assayed for radioactivity and enzymic activity.

Leydig cells were prepared from collagenase (Worthington) dispersed testis as described earlier [7].

3. Results

An accurate measurement of the amount of radioactivity taken up by testes under in vivo conditions is difficult because the blood cannot be readily removed from the tissue. To avoid this problem, all the experiments were performed using a 27 000 × *g* (15 min) pellet which was found to contain 97% of the radioactivity associated with testicular particulate material 15 min after an injection of 50 μg [*methyl*-^{3}H]lutropin into mature rats. The 27 000 × *g* (15 min) pellet contained 40% of the protein, 80% of the acid phosphatase activity (marker for lysosomes) and 60% of the 5′-nucleotidase activity (marker for plasma membranes), relative to the testicular homogenate. The radioactivity associated with this particulate fraction accounted for 30–50% of that present in the testicular homogenate, and it can be competitively diluted out by injecting [*methyl*-^{3}H]lutropin simultaneously with unlabeled hormone as shown in table 1. The association of the hormone with this fraction is thus considered specific. Most of the radioactivity that is not associated with the particulate fraction is TCA-soluble and is believed to arise mainly from the trapped blood which contains tritiated amino acids from non-target tissue catabolism of [*methyl*-^{3}H]lutropin [7,8].

Figure 1A shows the total radioactivity associated with the testicular particulate fraction at various times after injection of [*methyl*-^{3}H]lutropin. The

Table 1
Competition of the testicular uptake (particulate fraction) of tritiated lutropin by unlabeled hormone[a]

Lutropin injected (μg)	dpm/pellet[b]
0	6640
10	3152
90	800

[a] In all cases 10 μg of [*methyl*-^{3}H]lutropin were injected simultaneously with lutropin. The animals were sacrificed 15 min after injection and the testes were removed, homogenized, and centrifuged at 27 000 × *g* for 15 min. The pellet was resuspended in 5 ml 0.25 M sucrose and 0.5 ml aliquots were counted.

[b] Each pellet contained approx. 30 mg protein.

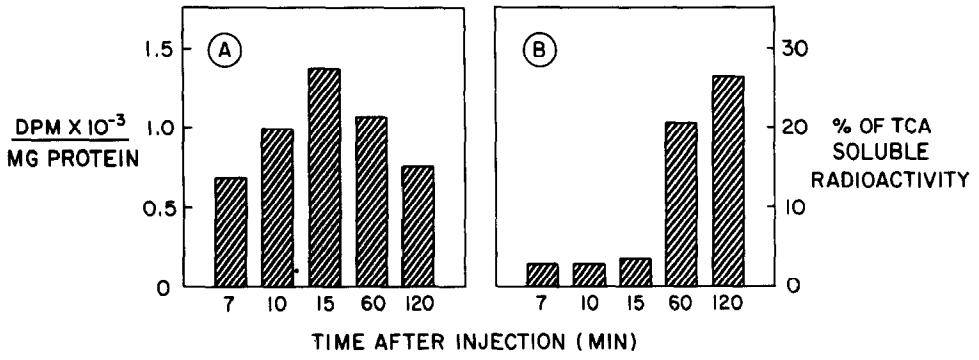

Fig.1. Target tissue uptake of lutropin; rats were injected with 50 μg [*methyl*-^{3}H]lutropin at time zero. The testicular 27 000 × *g* (15 min) pellet was homogenized and the total and TCA-soluble radioactivity was determined. (A) The total radioactivity at various times post-injection. (B) Percentage of the total radioactivity in (A) that is TCA-soluble.

maximum appears to occur at about 15 min. This is similar to that observed in liver and kidneys [6–8]. Between 15 min and 60 min, the amount of TCA-soluble radioactivity in this fraction increased from less than 4% to greater than 20% of the total particulate-associated radioactivity (fig.1B). It is noteworthy that this increase occurs during a time interval in which the total radioactivity is diminishing. The TCA-soluble radioactivity corresponds both to single amino acids and oligopeptides as shown by copper–Sephadex chromatography (fig.2). These

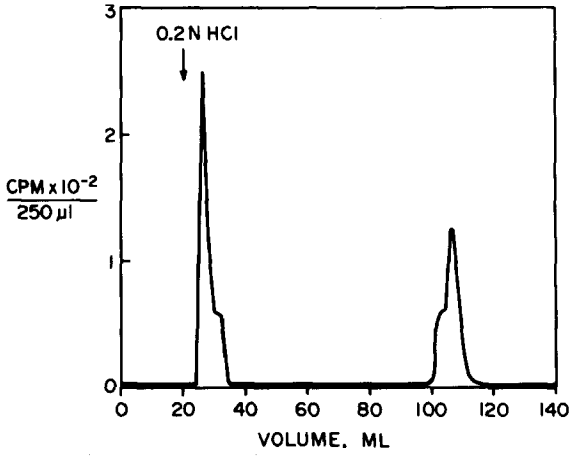

Fig.2. Analysis of the TCA-soluble products from the testicular 27 000 × *g* (15 min) particulate fraction, obtained 60 min after injection of [*methyl*-^{3}H]lutropin, using copper–Sephadex chromatography. Peptides elute between 25–35 ml and amino acids elute between 100–115 ml.

results demonstrate that testicular tissue can degrade the hormone that is bound under in vivo conditions.

In an effert to separate the plasma membrane and lysosomal fractions, the testicular 27 000 × *g* (15 min) pellet was analyzed on a 17–56% sucrose-gradient which resolves these subcellular fractions in other tissues [17,25]. Based on the activity of acid phosphatase and 5′-nucleotidase, there was no evidence of separation of the two subcellular fractions in the testicular material. The activities of both enzymes, as well as the radioactivity at 5 min and 60 min following an injection of [*methyl*-^{3}H]lutropin, banded between the densities 1.16 g/ml and 1.22 g/ml [13].

Partial separation of the testicular plasma membrane and lysosomal fraction was accomplished with a 40–60% sucrose gradient. Two major protein bands were observed when the testicular pellet was analyzed on this gradient. The first band occurred about 0.5 cm from the top of the gradient and contained most of the 5′-nucleotidase activity. The second band was located about 7 cm from the top and contained most of the acid phosphatase activity. The results, obtained by first preparing Leydig cells and then the particulate fraction, are shown as a density distribution [17,25] in fig.3 along with the density distribution of radioactivity 5 min and 60 min after injection of [*methyl*-^{3}H]lutropin. With increasing time after injection of the labeled hormone, there is a migration of radioactivity toward higher density suggesting a translocation from the plasma membrane fraction to the lysosomal fraction. A similar movement of radio-

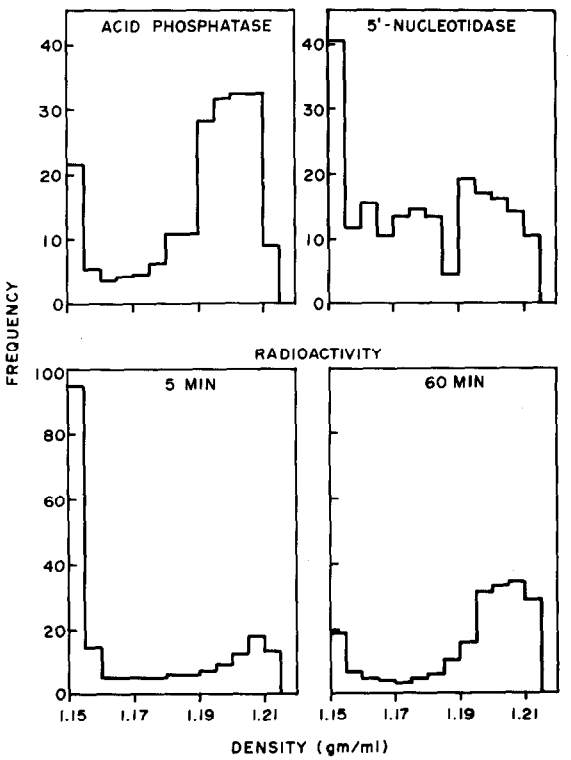

Fig.3. Subfractionation of the Leydig cell 27 000 × *g* (15 min) pellet on a 40–60% sucrose-gradient. The density distribution of acid phosphatase and 5′-nucleotidase is shown in the upper panels. These represent the average of 2 gradients and material from 2 animals was used in each gradient. The lower panels show the density distribution of radioactivity 5 min and 60 min after injection of 50 μg [*methyl*-^{3}H] lutropin.

activity with time after injection of [*methyl*-^{3}H] lutropin was observed using a discontinuous sucrose gradient of the (total) testicular pellet and the results are summarized in table 2.

The structural latency of lysosomal enzymes can be released by various treatments [13,14,17,24,25]. Several of these treatments have been used on the testicular particulate fraction, isolated 5 min and 60 min after injection of [*methyl*-^{3}H]lutropin, and the results are summarized in table 3. It can be seen that 2–3 times more radioactivity can be released from the 60 min fraction than from the 5 min fraction. These results are consistent with the hypothesis that the lysosomes are responsible for catabolizing lutropin in male target-tissue.

Table 2
Relative distribution of acid phosphatase, 5′-nucleotidase and radioactivity from the testicular particulate fraction in a discontinuous sucrose-gradient[a]

Constituent at sucrose-interface	Relative activity[b]	
	(Fractions 1–3)	(Fractions 7–9)
Enzymic activity:		
Acid phosphatase	0.88	1.06
5′-Nucleotidase	1.56	0.74
Radioactivity:		
5 min	1.34	0.83
60 min	0.72	1.13

[a] The testicular 27 000 × *g* (15 min) pellet was analyzed using the discontinuous sucrose-gradient as described in Materials and methods.

[b] The values given represent the relative amount of the constituent at each sucrose-interface calculated as follows. Enzymic activities, radioactivities and total protein in pooled fractions 1–3 and pooled fractions 7–9 were each normalized to unity and the values given represent the enzymic- or radioactivity relative to the protein content. The protein content of combined fractions 7–9 was about twice that of combined fractions 1–3. At time zero, 50 μg [*methyl*-^{3}H]lutropin was injected and one group of animals was sacrificed 5 min later and another group 60 min later.

4. Discussion

Our results are consistent with the following model. After binding of lutropin to a specific receptor on the Leydig cell plasma membrane and (probable) subsequent cellular activation, the hormone–receptor complex is internalized, presumably via endocytosis,

Table 3
Release of radioactivity from the testicular 27 000 × *g* (15 min) pellet 5 min and 60 min after injection of 50 μg [*methyl*-^{3}H]lutropin

Treatment[a]	Radioactivity released (% total)	
	5 min	60 min
Osmotic shock	9.8	30.0
0.1% (w/v) Triton X-100	25.0	52.0
Homogenization	6.0	21.0

[a] Each of these treatments released 95–100% of the acid phosphatase activity and 2–5% of the 5′-nucleotidase activity

and the endocytic vesicles fuse with primary lysosomes where catabolism then occurs. There is no firm evidence on the fate of the receptor. The suggestion that it may also be internalized is based on the following information. Binding of lutropin to the plasma membrane of target-tissue (i.e., Leydig cells in males) seems to be a prerequisite for internalization; moreover, the membrane surrounding the endocytic vesicle is believed to arise from the plasma membrane [26,27]. Also, Hsueh et al. found that the presence of gonadotropins leads to an apparent decrease in the number of receptors [5].

Midgley and coworkers have proposed a similar model to explain their autoradiographic results on the in vivo interaction of iodinated human choriogonadotropin with the ovaries of pseudopregnant rats [28]. However, their observations could not be confirmed by following the distribution of radioactivity and subcellular organelles by sucrose-gradient centrifugation since a good separation was not achieved between the plasma membrane and lysosomal fractions [29]. A similar mechanism was also proposed to explain the interaction of epidermal growth factor with cultured human fibroblasts [30]. Lastly, the observations that the number of receptor sites for growth hormone and insulin in cultured human lymphocytes are regulated by the extracellular concentration of the hormones [31,32] and the apparent regulation of the number of receptors in pituitary cells by thyrotropin releasing factor [33], are consistent with this model.

The proposed model provides an efficient and general pathway by which the action of peptide and glycoprotein hormones can be terminated. No information is available on whether the process of internalization has associated biological actions, or if it serves mainly to terminate hormone action and to regulate the number of receptors.

Acknowledgements

This work was supported by Research Grant AM-15838. The Center for Population and Reproductive Biology Research (HD-05797) and the Population Training Grant (HD-07043) also provided valuable services. D.P. is the recipient of a Research Career Development Award (AM-00055) and a Dreyfus Teacher-Scholar Award. It is a pleasure to thank Mr Rodger A. Liddle for expert assistance.

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