Evidence for the rapid internalization and recycling of lutropin receptors in rat testis Leydig cells

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A study into the binding of ^{125}I -human chorionic gonadotropin (hCG) to the lutropin (LH) receptor in rat testis Leydig cells, and subsequent internalization of the hormone–receptor complex, has been carried out. The results show that there is rapid internalization of the hormone–receptor complex; 240 receptors/cell (from a total of approx. 4000 receptors/cell) were internalized each minute in the first hour after exposure to hCG. Radioactivity was released from the cell 1 h after internalization and was found to be associated with highly degraded hCG. The endocytic process was found to have two temperature-sensitive steps. At 4 °C, movement of the hormone–receptor complex inside the cell did not occur, and at 21 °C hormone accumulated within the cytoplasm but was not degraded or released from the cell. At 34 °C, internalization, degradation and loss of the degraded hormone from the cell occurred. These processes appeared to reach a steady state after 2 h. Even though there is rapid internalization of the hormone–receptor complex following exposure to hCG, the binding sites on the cell surface were maintained for at least 4 h. The number of binding sites on the cell surface was not decreased by a protein synthesis inhibitor but was reduced to undetectable levels by monensin. This compound inhibits acidification of endocytic vesicles, which is known to be an important prerequisite to receptor cycling. It is concluded that, in the rat testis Leydig cells, following binding of hCG to the LH receptor there is rapid internalization of the complex and that recycling of the receptor occurs to the cell surface. This process may be essential in maintaining the capacity of the Leydig cell to bind fresh hormone.

INTRODUCTION

It is established that, in many adenylate cyclase systems, following stimulation of cyclic AMP production there is a time- and dose-dependent uncoupling of the hormone receptor from the adenylate cyclase, so that the cells become resistant, i.e. desensitized, to further stimulation. This lesion or uncoupling has been proposed as the first step in the clustering and internalization of the hormone–receptor complex, most notably in the β -adrenergic system (Strulovici *et al.*, 1983; Toews & Perkins, 1984; Stadel *et al.*, 1983). The process of internalization has been demonstrated visually, either with fluorescent derivatives of the ligands, or by using electron microscopic autoradiographic techniques. The hormones studied include insulin (Schlessinger *et al.*, 1978; Carpentier *et al.*, 1978), epidermal growth factor (Gorden *et al.*, 1978), transferrin (Harding *et al.*, 1983) and thyroid stimulating hormone (Avivi *et al.*, 1981, 1982). After the hormone–receptor complexes have clustered in clathrin-coated pits these invaginate and eventually form coated vesicles which move into the cytoplasm of the cell (Willingham & Pastan, 1983; Nilsson *et al.*, 1983; Harding *et al.*, 1983). There is then in most systems a ‘sorting process’ whereby receptors and hormones are dissociated by acidification of the vesicles (Tycko & Maxfield, 1982; Forgac & Cantley, 1984; Wolkoff *et al.*, 1984) and then separated into two endosomal compartments. The ligand is degraded in the lysosomes and the products subsequently released (Harford *et al.*, 1983; Ascoli & Puett, 1978), whilst there is strong evidence that the receptors are returned to the

cell surface (Hertel & Staehelin, 1983; Schwartz *et al.*, 1984).

In glycoprotein hormone sensitive systems such as the testis Leydig cell, it has been shown that, during short term incubation with LH, there is no net loss of the LH receptors whilst there is desensitization. It has been established by Dix *et al.* (1982), using the rat tumour Leydig cell, that the lesion responsible for desensitization of adenylate cyclase lies between the receptor and the GTP-binding protein (N_s protein) of the adenylate cyclase system. Catt *et al.* (1979), using experiments *in vivo* with hCG, refer to an occupancy stage where there is little or no net loss of LH receptors in Leydig cells and which lasts up to 24 h. This is then followed by a prolonged loss of receptors which is maximal after 2 days. Thus down-regulation of the LH receptor and early desensitization of the Leydig cell did not appear to be connected (Dix *et al.*, 1982; Wu *et al.*, 1985). However, it was assumed that there is little movement or turnover of LH receptors occurring in the Leydig cell, because no net loss in their number during short term studies could be measured, and this does not take into account the existence of an endocytic pathway leading to recycling of receptors.

The purpose of this study was to investigate the dynamics of LH receptor binding, internalization and recycling in purified rat testis Leydig cells. This has been carried out using ^{125}I -labelled hCG and by studying the effects of monensin, which inhibits acidification of endocytic vesicles and thus prevents intracellular hormone–receptor dissociation. The results obtained indicate that LH receptors are rapidly internalized and inserted

Abbreviations used: hCG, human chorionic gonadotropin; LH, lutropin.

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into the plasma membrane, possibly by recycling, and also that the internalized hormone is degraded and excreted.

Part of this work has been presented at the 613th meeting of the Biochemical Society (Habberfield *et al.*, 1985).

MATERIALS AND METHODS

Materials

hCG (NIH CR121) was obtained from the Endocrinology Study Section, National Institutes of Health, Bethesda, MD, U.S.A. Bovine serum albumin (fraction V), Hepes and monensin were purchased from Sigma Chemical Co., and Percoll was from Pharmacia Fine Chemicals. Na¹²⁵I (carrier-free) was obtained from The Radiochemical Centre, Amersham, Bucks, U.K. Eagle's minimum essential medium without NaHCO₃ and penicillin/streptomycin were purchased from Gibco, Paisley, Scotland, U.K.

Iodination procedure

hCG and ovalbumin were radioiodinated by the lactoperoxidase method and purified by Sephadex G-100 chromatography essentially as described by Thorell & Johansson (1971). Characterization of the ¹²⁵I-labelled hCG was by a competitive binding assay with hCG (NIH CR121).

Cell preparation and culture

Leydig cells were isolated and purified on 0–90% Percoll gradients as previously described (Hunter *et al.*, 1982; Aldred & Cooke, 1982). These were plated out in Costar wells at a cell density of 50 000–100 000 cells/well in Eagles' minimum essential medium containing bovine serum albumin (0.1%), Hepes (10 mM) and penicillin/streptomycin (50 units/ml), pH 7.4. After plating out, the cells were always preincubated for at least 1 h. For all binding studies, cells were incubated with ¹²⁵I-labelled hCG (50 ng/ml; sp. radioactivity 3×10^7 d.p.m./ μ g). Non-specific binding was estimated in the presence of excess hCG (400 i.u./ml). When indicated, various experimental conditions were altered, including different temperatures, preincubation with hormone and the addition of monensin.

Determination of surface binding and internalization

At the end of the incubation period the Costar wells were placed on ice and the media were removed for determination of total and trichloroacetic acid-soluble radioactivity released. The cells were washed in buffer containing sodium phosphate (0.01 M), NaCl (0.9%), bovine serum albumin (0.2%), methyl cellulose (0.1%) pH 7.4, and then incubated for 2 min at 4 °C in acidified buffer containing glycine (50 mM) and NaCl (0.9%), pH 3.0. This was then removed and the cells were washed once with 0.5 ml of acid buffer. This method is essentially as described by Ascoli (1982). The two acid washes were combined and counted to give acid-sensitive surface-bound radioactivity. The cells were washed once more in pH 7.4 buffer prior to being dissolved by incubation at room temperature for 30 min in 0.1 M-NaOH. This was then counted to give the acid-resistant internalized radioactivity.

Determination of the total radioactivity released

Fig. 1. Surface binding and internalization of ¹²⁵I-hCG in rat testis Leydig cells at 34 °C

Rat testis Leydig cells were prepared as described in the Materials and methods section and then incubated in the presence of ¹²⁵I-labelled hCG at 34 °C. Surface-bound (●) and internalized (○) levels were determined by acid washing as described in the Materials and methods section. All points are corrected for non-specific binding, and are means $\pm$ S.E.M. (represented by the bars) for triplicate determinations for each time point.

(which represents degraded and undegraded hCG) and trichloroacetic acid-soluble release (degraded hCG) were as follows and essentially as described by Ascoli & Puett (1978). Any whole cells were removed from the media by centrifugation; the media were then incubated with an equal volume of 20% (w/v) trichloroacetic acid at 4 °C for 1 h. Precipitated proteins were pelleted by centrifugation at 1200 g for 15 min and counted. The difference between total and precipitated radioactivity was taken as acid-soluble highly degraded hormone.

RESULTS

Internalization of ¹²⁵I-hCG by rat testis Leydig cells at 34 °C was found to be a rapid process (Fig. 1). In the presence of 50 ng of ¹²⁵I-hCG/ml, internalization occurred at a rate of 0.4 fmol/min. Over the same period of time the surface binding appeared to increase at a slower rate of 0.06 fmol/min, and unlike internalized levels it did not reach a plateau (Fig. 1).

At 4 °C there was no significant increase in internalized levels of hormone even up to 20 h of exposure to ¹²⁵I-hCG (Fig. 2). Surface binding, however, increased to a level of 6.75 fmol/10⁶ cells after 4 h and remained at that level for up to 20 h. A 6 h incubation with ¹²⁵I-hCG in the

Fig 2. Surface binding and internalization of ^{125}I -hCG in rat testis Leydig cells at 4 °C

Leydig cells were prepared as described in the Materials and methods section and then incubated in the presence of ^{125}I -labelled hCG at 4 °C. Surface binding (●) and internalized (○) levels were determined by acid washing as described in the Materials and methods section. All points are corrected for non-specific binding, and are means $\pm$ S.E.M. (represented by the bars) for triplicate determinations for each time point.

Table 1. Effect of NaN_3 on internalization of ^{125}I -hCG in rat testis Leydig cells

Rat testis Leydig cells were prepared as described in the Materials and methods section and then incubated in the presence of ^{125}I -labelled hCG (50 ng/ml; sp. radioactivity 3×10^7 d.p.m./ μg) and NaN_3 (5.0 mM) at 34 °C. Surface binding and internalized levels were determined by acid washing as described in the Materials and methods section. All points are corrected for non-specific binding, and are the means $\pm$ S.E.M. for triplicate determinations for each time point.

Time (h)	^{125}I -hCG (fmol/ 10^6 cells)	
	Surface-bound	Internalized
1	2.27 ± 0.26	0.26 ± 0.03
2	5.9 ± 0.2	0.9 ± 0.1
4	5.76 ± 0.4	1.75 ± 0.06
6	4.96 ± 0.8	1.79 ± 0.06

presence of 5 mM- NaN_3 inhibited internalization of the hormone (Table 1). This was most effective at 2 h; 5.9 fmol of ^{125}I -hCG/ 10^6 cells (86% of radioactivity) was bound to the surface of the cells and only 0.9 fmol (13% of total bound) was internalized.

To assess the 'non-specific' uptake of proteins into the Leydig cells, the binding and internalization of iodinated ovalbumin, which has a similar molecular size to hCG (44 and 38 kDa respectively), were investigated. At 34 °C the

Table 2. Apparent binding and acid-resistance of ^{125}I -labelled ovalbumin and ^{125}I -hCG in rat testis Leydig cells

Rat testis Leydig cells were prepared as described in the Materials and methods section and then incubated for the times shown in the presence of ^{125}I -labelled hCG (1×10^6 c.p.m./ml) or ^{125}I -labelled ovalbumin (1×10^6 c.p.m./ml) at 34 °C. Acid washing was carried out as described in the Materials and methods section. Non-specific binding for ^{125}I -hCG was described in the Materials and methods section, and for ^{125}I -ovalbumin an excess (1 $\mu\text{g}/\text{ml}$) of ovalbumin was added to the cells. All points are corrected for non-specific binding, and are the means $\pm$ S.E.M. for triplicate determinations for each time point.

Time (h)	^{125}I (c.p.m./ 10^6 cells)	
	Acid-susceptible	Acid resistant
^{125}I -hCG	0	0
	1	12177 ± 790
	2	24820 ± 1693
	4	27071 ± 730
^{125}I -Ovalbumin	0	703 ± 392
	1	475 ± 355
	2	2531 ± 2354
	4	746 ± 187

levels of 'acid-resistant' radioactivity of the iodinated ovalbumin increased up to 11277 c.p.m. from 5×10^5 c.p.m. added, compared with 31765 acid-resistant c.p.m. for ^{125}I -hCG (Table 2). At the same time surface-bound radioactivity was 746 c.p.m. and 27071 c.p.m. for ^{125}I -ovalbumin and ^{125}I -hCG respectively.

In order to study the release of radioactivity (from partially or fully degraded internalized ^{125}I -hCG), the cells were preincubated with ^{125}I -hCG at 4 °C for 2 h, and the hormone was then removed from the incubation medium by washing the cells with buffer. Following warming to 34 °C, release of radioactivity was found to occur from the cells after 60 min, after which time it rapidly increased (Fig. 3a). However, it was also found that, following the washing of the cells and warming to 34 °C, there was a very rapid release of radioactivity (equivalent to 2 fmol/ 10^6 cells), within the first 10 min of incubation. Since this level of release remained unchanged up to 60 min, this appeared to be the result of trauma due to the washing and rewarming rather than release as measured after 60 min. To measure ^{125}I -labelled acid-soluble release, the cells were treated in the same manner, except that the preincubation was at 34 °C for 1 h. The percentage of acid-soluble release increased from 37% to 82% of total release over the 4 h reincubation (Fig. 3b).

As previously shown in Fig. 2, incubation of Leydig cells at 4 °C inhibited the internalization of the hormone. Fig. 4(a) shows the effect of preincubation for 2 h in the presence of ^{125}I -hCG, followed by a further 4 h incubation after removal of the hormone from the medium, all at 4 °C; it can be seen that the surface binding remained at about 11 fmol/ 10^6 cells and there was no detectable increase in ^{125}I -labelled release. At 21 °C (Fig. 4b), over the 2 h preincubation, internalized levels rose to 25 fmol/ 10^6 cells and remained at that level for the subsequent 4 h incubation without hormone. Cell surface-bound levels dropped to insignificant levels after 1 h but then increased to 8 fmol/ 10^6 cells at 4 h. At the

Fig. 3. Degradation and release of ^{125}I -hCG in rat testis Leydig cells

Rat testis Leydig cells were prepared as in the Materials and methods section. (a) The cells were then preincubated for 2 h in the presence of ^{125}I -labelled hCG at 4 °C. After washing twice with fresh media, the cells were reincubated without hormone at 34 °C and media removed at the time points shown for determination of total radioactivity released ($\square$). (b) The cells were preincubated for 1 h in the presence of ^{125}I -labelled hCG at 34 °C. After washing twice with fresh media, the cells were reincubated at 34 °C without hormone, and media removed at the times shown for determination of total ($\square$) and acid-soluble ($\blacksquare$) radioactive release as described in the Materials and methods section. Non-specific release of radioactivity was determined as for the binding experiments. All points are corrected for this, and are the means $\pm$ S.E.M. (represented by the bars) for triplicate determinations for each time point.

same time there was an increase in released radioactivity, which rose to the equivalent of 6 fmol/10⁶ cells of ^{125}I -hCG. At 34 °C after a 2 h preincubation, levels of internalized hormone reached 35 fmol/10⁶ cells (Fig. 4c), compared with surface-bound levels of 10 fmol/10⁶ cells. Over the 4 h incubation, internalized levels fell to 7.5 fmol/10⁶ cells, a drop of 27.5 fmol/10⁶ cells, whilst acid-soluble release rose to an ^{125}I -hCG equivalent of 20.7 fmol/10⁶ cells. The surface-bound levels increased by 7 fmol/10⁶ cells.

The effect of monensin (which prevents acidification of endocytic vesicles) and cycloheximide (a protein synthesis inhibitor) on the internalization and surface binding of ^{125}I -hCG was investigated at 34 °C. Incubation with ^{125}I -hCG in the presence of 25 μM -monensin caused a large reduction in total binding of hormone to the cells compared with control (Fig. 5). In control cells, total binding reached 55 fmol/10⁶ cells after a 2 h incubation compared with 7 fmol/10⁶ cells in monensin-treated cells. Surface binding over this time was reduced to undetectable levels by monensin. There was no effect of monensin on binding of ^{125}I -hCG to the cells under control binding conditions at 4 °C (Table 3).

Treatment with cycloheximide did not remove all the surface binding as monensin had done, but did reduce total binding over a 2 h incubation to 29 fmol/10⁶ cells or 53% of control cell binding. The level of binding was reduced equally for both the surface-bound and internalized components. At 4 h of incubation, however, there was no difference in the levels of binding between control and cycloheximide-treated cells.

The effect of 25 μM -monensin on the degradation and release of internalized hormone in testis Leydig cells was

also examined (Fig. 6). In control cells over a 4 h incubation, total and acid-soluble release rose to an ^{125}I -hCG equivalent of 13 fmol/10⁶ cells and 7 fmol/10⁶ cells respectively. In monensin-treated cells, total release rose to an ^{125}I -hCG equivalent of only 5.5 fmol/10⁶ cells and none of this was acid-soluble, i.e. highly degraded, hormone.

DISCUSSION

The results obtained in this study demonstrate that binding of LH (hCG) to lutropin receptors in isolated rat testis Leydig cells leads to rapid internalization of the hormone-receptor complex. This appears to be followed by dissociation of the hormone-receptor complex in endocytic vesicles and the return of the receptors to the cell surface. During these short term incubations there is no net loss of receptors, i.e. down-regulation does not occur. However, contrary to previous results which indicated that the hormone-receptor complex was stable until after prolonged exposure to the hormone (Catt *et al.*, 1979), it is now apparent that the rat testis Leydig cell contains a highly dynamic internalization-recycling system.

Many receptors, after binding of a specific ligand, undergo endocytosis. In some cases transcytosis of the ligand occurs, whilst in others the ligand is degraded and released. The fate of the receptor is variable; recycling to the cell surface can occur, e.g. the transferrin receptor (Harding *et al.*, 1983) and the β -adrenergic receptor (Hertel & Staehelin, 1983), and down-regulation, i.e. actual loss of receptors, can occur as with the EGF receptor (Beguinot *et al.*, 1984). It has also been shown

Fig. 4. Effect of temperature on surface binding, internalization and degradation of ^{125}I -hCG in rat testis Leydig cells

Cells were prepared as in the Materials and methods section and then incubated at (a) 4 °C, (b) 21 °C or (c) 34 °C for 1 h. ^{125}I -labelled hCG was then added to the cells, which were incubated at the same temperatures for 2 h prior to washing twice with fresh media. The cells were reincubated without hormone at the same temperature. Determination of surface-bound (●), internalized (○) and acid-soluble release (■) were all as described in the Materials and methods section. All points are corrected for non-specific binding and are the means $\pm$ S.E.M. (no S.E.M. for the acid-soluble release) for triplicate determinations for each time point.

Fig. 5. Effect of monensin and cycloheximide on surface binding and internalization of ^{125}I -hCG in rat testis Leydig cells

Cells were prepared as described in the Materials and methods section, and then incubated for 30 min in the absence (a) or presence of monensin (25 μM) (b) or cycloheximide (2.5 $\mu\text{g/ml}$) (c) at 34 °C. ^{125}I -labelled hCG was then added to all the cells, which were further incubated for the times shown. Surface bound (●) and internalized (○) levels were determined as in the Materials and methods section. All points are corrected for non-specific binding and are the means $\pm$ S.E.M. (represented by the bars) for triplicate determinations for each time point.

in the MA 10 tumour Leydig cell that internalization of LH receptors does occur following binding of ^{125}I -hCG and that there is subsequent degradation and release of the ligand (Ascoli, 1982).

The rate at which receptor-ligand complexes are internalized varies with the cell type. Adipocytes (Reed *et al.*, 1984), hepatocytes (Wolkoff *et al.*, 1984) and others are rapid internalizers. The U-937 monocytes internalize

50% of bound ^{125}I -insulin within 2 min. Others such as IM-9 lymphocytes are slow internalizers; only 3% is internalized after 30 min (Carpentier *et al.*, 1984). In this study 77% of the cell-associated ^{125}I -hCG was internalized within 15 min, and an equivalent of 240 LH receptors/cell (from a total of approx. 4000) were internalized each minute in the first hour after exposure to the hormone. That this uptake of ^{125}I -hCG is a specific process is shown

Table 3. Effect of monensin on binding of ^{125}I -hCG to rat testis Leydig cells

Cells were prepared as in the Materials and methods section, then preincubated for 30 min at 34 °C in the absence or presence of monensin (25 μM). The cells were then incubated at 4 °C with ^{125}I -labelled hCG (50 ng/ml; sp. radioactivity 3×10^7 d.p.m./ μg) for the times shown. Binding was determined simply by washing the cells twice in phosphate/NaCl buffer, pH 7.4. The results are corrected for non-specific binding and are the means $\pm$ S.E.M. for triplicate determinations for each time point.

Time (h)	^{125}I -hCG (fmol/ 10^6 cells)	
	Control	+ Monensin (25 μM)
1	0.58 ± 0.1	1.56 ± 1.0
2	5.8 ± 1.5	4.39 ± 2.4
4	6.4 ± 1.0	5.7 ± 0.4
6	7.8 ± 0.26	7.9 ± 0.63

by the much slower rise in acid-resistant binding of ^{125}I -ovalbumin, although by 2 h the radioactivity of ^{125}I -ovalbumin had risen to a third of that for ^{125}I -hCG. It may be, however, that because ovalbumin is being taken up non-specifically, as shown by the lack of surface binding, it is not being degraded and lost from the cells in the same manner as ^{125}I -hCG.

That internalization of the ^{125}I -hCG receptor complex is a dynamic process was demonstrated by incubation of the testis Leydig cells at 4 °C. At this temperature the binding sites were saturated by 4 h after exposure to the hormone, compared with 2 h at 34 °C. The level of binding, however, was much lower at 4 °C and remained surface-bound up to 20 h after exposure to the hormone. The energy-dependence of LH receptor internalization was shown by incubation of testis Leydig cells with ^{125}I -hCG in the presence of NaN_3 at 34 °C. This compound is known to deplete the cells of ATP and was found to inhibit the internalization of the LH receptors.

Previous studies by Ascoli & Puett (1978) in the murine tumour Leydig cell have shown that internalized ^{125}I -hCG is degraded and released as mono[^{125}I]iodotyrosine. There is strong evidence that the lysosomes are involved in breakdown of the ligand in this and other systems, including platelet-derived growth factor in fibroblasts (Nilsson *et al.*, 1983). In the testis Leydig cell we have shown that release of ^{125}I -labelled degradation products begins 1 h after the internalization of ^{125}I -hCG. A large part of this release is acid-soluble, indicating that it is highly degraded hormone.

The large level of internalization which occurs in the 1 h after exposure to hormone, compared with the delay in release of degraded hormone, suggests that at least some of the cell-associated hormone measured after incubation at 34 °C is not receptor-bound. This hormone may well remain in the endocytic vesicle and lysosomes of the cell as part of its 'processing', prior to the release of the degradation products. This could have important implications in the determination of receptor affinity and number, using Scatchard analysis of whole cells. It is also interesting to note that after 2 h at 34 °C, where the level of binding appears to reach a plateau, must represent a

Fig. 6. Effect of monensin on total and acid-soluble ^{125}I -labelled release in rat testis Leydig cells

Cells were prepared as in the Materials and methods section and then pre-incubated in the absence (a) or presence (b) of monensin (25 μM) for 60 min, and then for a further 90 min with ^{125}I -labelled hCG. The cells were then washed twice with either media alone (a) or media containing monensin (25 μM) (b) and then further incubated in these respective media for the time shown. Determination of total release ($\square$) and acid-soluble release ($\blacksquare$) were all as described in the Materials and methods section. All the points are corrected for non-specific release and are the means $\pm$ S.E.M. (represented by the bars) for triplicate determination for each time point.

point where internalization into the cell and release of degraded hormone from the cell have reached a steady state, rather than saturation of the binding sites.

The effect of temperature on the endocytic pathway was investigated, using a similar technique to the measurement of release, in that after a 2 h preincubation with ^{125}I -hCG the cells were washed free of unbound hormone and reincubated without hormone so that movement of the hormone-receptor complex within the cell and release of degraded products could be followed. The results shown in Fig. 4 show two temperature-sensitive steps, firstly at the level of internalization, where a temperature of 4 °C does not affect binding but completely inhibits movement of the complex inside the cell and therefore any acid-soluble release is also blocked. At 21 °C in the testis Leydig cell the hormone-receptor complex moves into the cell cytoplasm from the cell surface but the degradation of the hormone is largely inhibited. There is therefore an accumulation of ^{125}I -labelled hormone within the cell, as found by Hopkins & Trowbridge (1983) in human carcinoma A431 cells. Wolkoff *et al.* (1984) also found two temperature-sensitive steps in the endocytic pathway of asialoglycoproteins and their receptors in rat hepatocytes. At 34 °C the endocytic pathway functioned completely in the testis Leydig cell, where a large drop in internalized levels of hormone over 4 h was accompanied by an increase of

acid-soluble release. Surface-bound levels of hormone, however, did not drop but actually increased by 7 fmol/10⁶ cells, this being the difference between total internalized loss and measurable acid-soluble release. It may be that this increase in surface binding is due to re-binding of partially degraded hormone to the receptors, but that this inactive form of the hormone is unable to stimulate internalization of the hormone-receptor complex.

If the Leydig cell internalizes 240 receptors/min, as determined from Fig. 1, we would expect that, in view of the limited number of receptors available, surface binding would be lost in the first 16 min after exposure to the hormone. Therefore to account for the continued increase in surface binding that is normally seen and the increase in internalized levels, one must determine whether the cell is synthesizing new receptor or if receptor recycling is occurring. Monensin, a carboxylic ionophore (for review see Pressman, 1976), has been used by many investigators to examine the mechanism of receptor return. It has been used to interrupt endosomal acidification (Yamashiro *et al.*, 1983), which is a necessary prerequisite for hormone-receptor dissociation (Wolkoff *et al.*, 1984). Incubation of the testis Leydig cells with monensin reduced total binding of ¹²⁵I-hCG by 65% after 2 h of incubation as compared with control cells with the largest relative decrease being in surface-bound levels. Monensin did not however affect binding of hormone to the receptor at 4 °C. These effects are similar to those seen by Wileman *et al.* (1984) with mannose glycoprotein receptors in the macrophage, where it was found that this reduction in binding was due to entrapment of receptor-ligand complexes in pre-lysosomal compartments and was linked to an increase in the pH of acidified intracellular vesicles. It is interesting to note that, in the testis Leydig cell in the presence of monensin, internalization remains rapid over the 1 h after exposure to hormone, but reaches a plateau much more quickly. This would be expected if the number of available receptors was limited by their inability to dissociate from the ligand and therefore to be recycled to the surface of the cell. It has been shown that monensin does not inhibit protein synthesis at concentrations up to 50 µM (Grinde, 1983; Rustan *et al.*, 1985), and therefore should not affect any possible receptor synthesis. It has been suggested, however, that continued surface binding where internalization or down-regulation is occurring, especially in long-term incubation, is dependent on the synthesis of new receptors (Mombrial *et al.*, 1984). If this were the case in the testis Leydig cell one would expect that in cycloheximide-treated cells by 4 h surface binding would be greatly reduced as compared with control cells. It was found, however, that the level of binding on the surface of the cell was no different from that of control cells and was more than twice the level seen in monensin-treated cells.

In summary, the data show that following binding of hormone to the LH receptor in the testis Leydig cell, the hormone-receptor complex is rapidly internalized into endosomal vesicles. These pass through a temperature-sensitive endocytic pathway and seem to be capable of acidifying their contents. This appears to be a necessary step prior to the recycling of the LH receptor to the cell surface. Following dissociation and segregation of the hormone-receptor complex, the hormone is degraded (which probably occurs in the lysosomes) and is

eventually released in a highly degraded form from the cell. Whether recycling of the LH receptor plays a part in the continued response of the Leydig cell to hormonal stimulation over relatively short periods of time has yet to be determined. It has previously been thought that LH receptors are in a large excess compared with the number needed to interact with hormone to elicit a steroidogenic response (Catt & Dufau, 1973). This, however, presumed that an endocytic pathway with receptor recycling was not occurring. The fact that the actual receptor number appears much lower than was previously thought may also have important implications in the relationship between receptor occupancy, cellular control of surface receptor number and the response of the cell to hormonal stimulation.

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