The post-endocytotic fate of the gonadotropin receptors is an important determinant of the desensitization of gonadotropin responses

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Abstract

Internalization of the ligand/receptor complexes is a consequence of the activation of the gonadotropin receptors. Since the recycling or degradation of the internalized receptors results in the maintenance or loss of cell surface receptors respectively and this contributes to the loss of responsiveness, we hypothesized that the fate of the internalized receptors could be an important component of desensitization. We examined this hypothesis using the wild-type and mutants of the human LH (hLHR) receptors and follitropin receptors expressed in MA-10 and KK-1 cells respectively. The receptor mutants were chosen because they are routed mostly to a lysosomal degradation pathway whereas the wild-type receptors are recycled back to the surface. We have shown that agonist stimulation of cells expressing the mutant receptors results in a more pronounced loss of cell surface receptors and agonist responses than stimulation of cells expressing the wild-type receptors. We concluded that receptor recycling promotes the maintenance of cell surface receptors and preserves hormonal responsiveness. This property of the hLHR is likely to be physiologically important because there at least two hLHR-expressing tissues in pregnant women, the maternal corpus luteum and the fetal Leydig cells, where a loss of hormonal responsiveness induced by the elevated levels of human chorionic gonadotropin that occur during pregnancy is not desirable.

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Introduction

The desensitization of lutropin (LH)/chorionic gonadotropin (CG)-induced responses has been studied in some detail in rodent granulosa and Leydig cells. Some of the hormone-induced effects that contribute to this phenomenon include: (a) an impairment in the functional properties of the cell surface LH receptor (LHR) that results in a decrease in adenylyl cyclase activation (Ekstrom & Hunzicker-Dunn 1989, Sánchez-Yagüe *et al.* 1993, Lamm & Hunzicker-Dunn 1994, Ascoli 1996, Wang *et al.* 1997, Mukherjee *et al.* 1999, Hunzicker-Dunn *et al.* 2002); (b) the down-regulation of cell surface receptors (Freeman & Ascoli 1981, Wang *et al.* 1991); (c) a decrease in cholesterol availability (Quinn *et al.* 1981, Freeman & Ascoli 1982); and (d) a decrease in the levels and/or activity of some of the steroidogenic enzymes such as 17 α -hydroxylase (Catt *et al.* 1980, Nozu *et al.* 1981).

The LH/CG-induced down-regulation of the cell surface LHR can be due to a decrease in the synthesis and/or an increase in the degradation of the LHR. In the MA-10 cells, we have shown that LH/CG-induced internalization and subsequent lysosomal degradation of the endogenous mouse LHR is the most important

contributor to down-regulation (Wang *et al.* 1991). LH/CG-induced changes in the synthesis of the LHR also occur. These are secondary to a cAMP-mediated decrease in the LHR mRNA but they are quantitatively unimportant to the overall process of receptor down-regulation (Wang *et al.* 1991, 1992).

During the past several years we (Ascoli 1984, Baratti-Elbaz *et al.* 1999, Kishi & Ascoli 2000, Kishi *et al.* 2001, Galet *et al.* 2003, 2004, Hirakawa *et al.* 2003, Krishnamurthy *et al.* 2003) and others (Ghinea *et al.* 1992, Beau *et al.* 1997, 1998, Baratti-Elbaz *et al.* 1999) have elucidated several features of the post-endocytotic trafficking of the porcine (p), rat (r), mouse (m) and human (h) LHR and follitropin receptors (FSHR). Most of the internalized hCG/rLHR, hCG/mLHR or hCG/pLHR complexes are routed to the lysosomes where the hormone and the receptor are degraded (Ascoli 1984, Ghinea *et al.* 1992, Baratti-Elbaz *et al.* 1999, Kishi *et al.* 2001). In contrast, most of the internalized hFSH/rFSHR or hFSH/hFSHR complexes accumulate in endosomes and subsequently recycle back to the cell surface where the bound, intact hFSH dissociates back into the medium and can bind to the receptor again (Krishnamurthy *et al.* 2003). The fate of the hCG/hLHR complex appears to be intermediate

between the fates of the complexes described above. In this case, there is little accumulation of the internalized hLHR in the lysosomes and there is a fairly even distribution in the amounts of internalized hCG that are released back into the medium in degraded and undegraded forms (Kishi *et al.* 2001, Galet *et al.* 2003, 2004, Hirakawa *et al.* 2003).

Since the recycling or degradation of the internalized gonadotropin receptors results in the maintenance or loss of cell surface receptors respectively (Wang *et al.* 1991, Kishi *et al.* 2000, 2001, Galet *et al.* 2003, 2004, Hirakawa *et al.* 2003, Krishnamurthy *et al.* 2003) and the loss of cell surface receptors contributes to desensitization (Wang *et al.* 1991), we hypothesized that the intracellular fate of the internalized receptors should be an important component of desensitization. The differential post-endocytotic fate of the rLHR and the hLHR (see above) could be a functionally important property of the hLHR because there at least two LHR-expressing tissues in pregnant women, the maternal corpus luteum and the fetal Leydig cells, where a loss of hormonal responsiveness induced by the elevated levels of hCG that occur during pregnancy is not desirable.

The experiments presented here were designed to test this hypothesis by examining the hormone-induced loss of cell surface receptors and responsiveness in target cells transiently transfected with the wild-type hLHR and hFSHR as well as in target cells expressing mutants of the hLHR and hFSHR that are targeted to a degradation pathway.

Materials and methods

Plasmids and cells

The preparation and characterization of expression vectors for the myc-hLHR-wt, myc-hLHR-t682, myc-hFSHR-wt and myc-hFSHR-t678 have been described (Min & Ascoli 2000, Hirakawa *et al.* 2003, Krishnamurthy *et al.* 2003). The origin, handling and methods used for transfection of MA-10 cells have been described (Ascoli 1981, Hirakawa *et al.* 2002, Hirakawa & Ascoli 2003a). The only change made is that the cells are now maintained in RPMI-1640 instead of Waymouth's MB752/1. (This change was prompted by the fact that most suppliers are no longer offering Waymouth's MB752/1.) A transformed mouse granulosa cell line (designated KK-1, see Rahman & Huhtaniemi 2001) was provided by Dr I Huhtaniemi (Imperial College, London, UK). KK-1 cells were maintained in Dulbecco's modified Eagles' medium modified to contain 10 mM Hepes, 10% newborn calf serum and 50 µg/ml gentamicin. These cells were transfected using the same procedures as those used for

Figure 1 The hCG-induced loss of cell surface hLHR is more pronounced in MA-10 cells expressing the hLHR-t682 than in MA-10 cells expressing the hLHR-wt. MA-10 cells transiently transfected with the hLHR-wt or hLHR-t682 were incubated with the indicated concentrations of hCG for 16 h at 37 °C. The cells were then washed with a neutral and an acidic buffer to remove the free and bound hCG and then they were used to measure residual ¹²⁵I-hCG binding during an overnight incubation at 4 °C as described in Materials and methods. Each point shows the mean ± S.E.M. of three to five independent transfections. The asterisks indicate a statistically significant difference (*t*-test, *P* ≤ 0.05) between the two groups of cells. The numbers above the last point depict ¹²⁵I-hCG binding as % of that detected in the cells incubated without hCG.

MA-10 cells (Hirakawa *et al.* 2002, Hirakawa & Ascoli 2003a).

Binding assays

Transiently transfected cell monolayers (in 35 mm wells) were washed and placed in 1 ml assay medium (RPMI-1640 containing 20 mM Hepes, 50 µg/ml gentamicin and 1 mg/ml bovine serum albumin (BSA), pH 7.4) and incubated for 16 h in the absence or presence of hFSH or hCG (30 nM). At the end of this incubation the cells were placed on ice and washed two to three times with 2 ml portions of cold wash medium (Hank's balanced salt solution containing 1 mg/ml BSA). The surface-bound hormone was then released by incubating the cells in 1 ml cold 50 mM glycine, 150 mM NaCl, pH 3 for 2–4 min (Ascoli 1982). This buffer was removed and the cells were washed once more with the same acidic buffer and then once with cold assay medium. The cells were then put back in 1 ml warm assay medium and incubated with ¹²⁵I-hCG or ¹²⁵I-hFSH (3 nM) overnight at 4 °C. At the end of this incubation the cells were washed three to four times (using cold Hank's balanced salt solution supplemented with 1 mg/ml BSA). After aspiration of the last wash, the cells were dissolved in 50 µl 1 M NaOH, collected

Figure 2 The hCG-induced loss of cAMP responses is more pronounced in MA-10 cells expressing the hLHR-t682 than in MA-10 cells expressing the hLHR-wt. MA-10 cells transiently transfected with the hLHR-wt or hLHR-t682 were incubated with 30 nM hCG for 16 h at 37 °C. The cells were then washed with a neutral and an acidic buffer to remove the free and bound hCG and then incubated without (open bars) or with 0.3 nM hCG (solid bars) for 30 min prior to the measurement of intracellular cAMP. Each bar shows the mean $\pm$ S.E.M. of three to five independent transfections. The numbers above the bars of the down-regulated cells display the hCG-induced cAMP response expressed as % of the response of the control cells expressing the same receptor. This was calculated after subtraction of the basal from the stimulated levels of cAMP in the control and down-regulated cells. The asterisk indicates a statistically significant difference (*t*-test, $P \leq 0.05$) between the two groups of down-regulated cells.

with a cotton swab and counted in a gamma counter. Three wells were used for each binding assay. Two of them received ^{125}I -hCG only but the third one also received 500 ng/ml crude hCG or 500 ng/ml equine FSH. This last well was used to correct for non-specific binding.

Cyclic AMP and progesterone assays

Transfected cell monolayers were incubated with or without gonadotropins for 16 h as described above. After removal of the surface-bound hormone (see above) the cells were put back in warm assay medium and incubated with or without hormone for 30 min (for cAMP assays) or 4 h (for progesterone assays) at 37 °C. Intracellular cAMP and extracellular progesterone were measured as described earlier (Segaloff *et al.* 1981, Hirakawa *et al.* 2002).

Hormones and supplies

Purified hCG (AFP8456A) was purchased from Dr A Parlow of the National Hormone and Pituitary Agency, Bethesda, MD, USA. Recombinant hFSH was kindly

provided by the Serono Reproductive Biology Institute (Rockland, MA, USA). ^{125}I -hCG and ^{125}I -hFSH were prepared as described elsewhere (Ascoli & Puett 1978). Cell culture supplies and reagents were obtained from Corning (Rochester, NY, USA) and Invitrogen (Carlsbad, CA, USA) respectively. All other chemicals were obtained from Sigma or Fisher Scientific (Pittsburgh, PA, USA). Radiochemicals were from Amersham.

Results

Down-regulation of the hLHR and hCG-mediated responses in transfected MA-10 cells

To assess the importance of receptor down-regulation on hCG responsiveness, we transiently expressed the hLHR-wt or an hLHR mutant truncated at residue 682 of the C-terminal tail (designated hLHR-t682) in MA-10 cells. Although MA-10 cells express endogenous mLHR, the expression of the transfected hLHR (or mutants thereof) is several orders of magnitude higher and, therefore, the functional properties of the transfected hLHR (or mutant thereof) can be readily examined

Figure 3 The hCG-induced loss of progesterone responses is more pronounced in MA-10 cells expressing the hLHR-t682 than in MA-10 cells expressing the hLHR-wt. MA-10 cells transiently transfected with the hLHR-wt or hLHR-t682 were incubated with 30 nM hCG for 16 h at 37 °C. The cells were then washed with a neutral and an acidic buffer to remove the free and bound hCG and then incubated without (open bars) or with 0.03 nM hCG (solid bars) for 4 h prior to the measurement of progesterone. Each bar shows the mean $\pm$ S.E.M. of three to five independent transfections. The numbers above the bars of the down-regulated cells display the hCG-induced progesterone response expressed as % of the response of the control cells expressing the same receptor. This was calculated after subtraction of the basal from the stimulated levels of progesterone in the control and down-regulated cells. The asterisk indicates a statistically significant difference (*t*-test, $P \leq 0.05$) between the two groups of down-regulated cells.

against a low background of endogenous mLHR (Hirakawa *et al.* 2002).

Figure 1 shows that MA-10 cells transfected with the hLHR-wt or hLHR-t682 bind similar levels of ¹²⁵I-hCG. Cells expressing either of these two receptors were incubated with increasing concentrations of hCG for 16 h at 37 °C, the free and surface-bound hormone were removed by washing the cells under neutral and acidic conditions respectively, and the residual cell surface receptors were measured using ¹²⁵I-hCG (Segaloff & Ascoli 1981, Ascoli 1982). The results presented in Fig. 1 show that at optimally effective concentrations of hCG (3–30 nM) there was a ~50% and ~75% loss respectively of surface receptors in cells expressing hLHR-wt or hLHR-t682. This difference in the hormone-induced loss of cell surface receptors has been previously described in a heterologous cell type (Hirakawa *et al.* 2003, Galet *et al.* 2004) and it is a reflection of the differential post-endocytotic fate of the hLHR-wt and hLHR-t682. Whereas roughly half of the internalized hCG/hLHR-wt complex is recycled back to the plasma membrane less than 10% of the internalized hCG/hLHR-t682 is recycled (Kishi *et al.* 2001, Galet *et al.* 2003, 2004, Hirakawa *et al.* 2003). The majority of

the hCG/hLHR-t682 complex is instead routed to a lysosomal degradation pathway where the hormone and the receptor are degraded (Kishi *et al.* 2001, Galet *et al.* 2003, 2004, Hirakawa *et al.* 2003).

To determine if these changes in receptor density have a differential effect on hCG responsiveness, MA-10 cells expressing the hLHR-wt or hLHR-t682 were incubated without or with 30 nM hCG for 16 h and the free and bound hormone were removed as described above. The control (i.e. cells treated without hCG) or down-regulated cells (i.e. cells treated with hCG) were then tested for their ability to respond with cAMP and progesterone accumulation to a new hCG challenge.

Transiently transfected MA-10 cells have a large complement of spare hLHR and the concentration-response curves for binding, cAMP and progesterone accumulation are characterized by EC₅₀ values of ~1, ~0.1 nM and ~0.03 nM respectively (Hirakawa *et al.* 2002). In this respect, the transiently transfected MA-10 cells resemble normal Leydig cells where steroid accumulation is maximal at low levels of receptor occupancy and cAMP accumulation (Mendelson *et al.* 1975). Therefore changes in steroid accumulation that may occur when receptor levels are reduced are more

Figure 4 The hFSH-induced loss of cell surface hFSHR is more pronounced in KK-1 cells expressing the hFSHR-t678 than in KK-1 cells expressing the hFSHR-wt. KK-1 cells transiently transfected with the hFSHR-wt or hFSHR-t678 were incubated with the indicated concentrations of hFSH for 16 h at 37 °C. The cells were then washed with a neutral and an acidic buffer to remove the free and bound hFSH and then they were used to measure residual ¹²⁵I-hFSH binding during an overnight incubation at 4 °C as described in Materials and methods. Each point shows the mean ± S.E.M. of three to five independent transfections. The asterisks indicate a statistically significant difference (*t*-test, *P* ≤ 0.05) between the two groups of cells. The numbers above the last point depict ¹²⁵I-hFSH binding as % of that detected in the cells incubated without hFSH.

likely to be detectable when cells are challenged with submaximal concentrations of hCG (Catt *et al.* 1979, 1980). As such, the residual responsiveness of control and down-regulated cells was measured using a nearly maximally effective concentration of hCG for cAMP accumulation (0.3 nM; Fig. 2) and a submaximal concentration of hCG for progesterone accumulation (0.03 nM; Fig. 3).

The results presented show that the residual cAMP (Fig. 2) and progesterone (Fig. 3) responses of down-regulated cells were proportional to the loss of receptors induced by pre-exposure to hCG. The left-hand panel of Fig. 2 shows that cells expressing equivalent levels of hFSHR-wt or hFSHR-t678 respond to an hCG challenge with a similar increase in cAMP accumulation. When these cells were pretreated overnight with hCG to induce receptor down-regulation (see right-hand panel of Fig. 2), however, the cAMP responsiveness of cells expressing the hFSHR-wt (where receptor loss was only ~50%, cf. Fig. 1) dropped to ~40% of the control cells. In contrast, the cAMP responsiveness of cells expressing the hFSHR-t678 and treated overnight with hCG (where receptor loss was ~75%, cf. Fig. 1) dropped to ~12% of the response of control cells.

A similar phenomenon was observed when progesterone was measured instead of cAMP (Fig. 3). The left-hand panel of Fig. 3 shows that cells expressing equivalent levels of hFSHR-wt or hFSHR-t678 responded to an hCG challenge with a similar increase in progesterone accumulation. When these cells were pretreated overnight with hCG to induce receptor down-regulation (see right-hand panel of Fig. 3), however, the progesterone responsiveness of cells expressing the hFSHR-wt (where receptor loss was only ~50%, cf. Fig. 1) dropped to ~50% of untreated cells. In contrast, the progesterone response of down-regulated cells expressing the hFSHR-t678 (where receptor loss was ~75%, cf. Fig. 1) dropped to ~30% of the response of control cells.

In contrast to the basal levels of cAMP, which increased minimally in the down-regulated cells (compare the open bars in the two panels in Fig. 2), the basal levels of progesterone were about 20-fold higher in the down-regulated cells than in the control cells (compare the open bars in the two panels in Fig. 3). This increase, which is due to the stimulation of the cells by the initial exposure to hCG, was similar in cells expressing either of the two receptors. Therefore the loss of responsiveness found in the down-regulated cells was due to a true differential decrease in the cAMP (compare the solid bars in the two panels of Fig. 2) or progesterone responses (compare the solid bars in the two panels of Fig. 3) to the new hCG challenge rather than to a differential increase in the basal levels of cAMP or progesterone induced by the previous treatment with hCG (compare the open bars in the two panels of Fig. 2 or Fig. 3).

Down-regulation of the hFSHR- and hFSH-mediated responses in transfected KK-1 cells

A similar set of experiments was then performed using a mouse granulosa cell line (KK-1 cells) transiently expressing the hFSHR-wt or a mutant truncated at residue 678 of the C-terminal tail. Untransfected KK-1 cells express small amounts of endogenous mFSHR as judged by their ability to bind ¹²⁵I-hFSH (data not shown). Since transient transfection of KK-1 cells with the hFSHR-wt or mutants thereof results in a five- to tenfold increase in ¹²⁵I-hFSH binding (data not shown), the transfected KK-1 cells can also be used to study the functional properties of the transfected FSHR against a low background of endogenous FSHR.

We have previously shown that, when expressed in several cell types (including KK-1 cells), most of the internalized hFSH/hFSHR-wt complex is recycled back to the plasma membrane and that truncation of the hFSHR at residue 678 re-routes a substantial portion of the internalized complex to a lysosomal degradation pathway (Krishnamurthy *et al.* 2003). In human kidney 293 cells, this re-routing of the hFSH/hFSHR-t678

Figure 5 The hFSH-induced loss of cAMP responses is more pronounced in KK-1 cells expressing the hFSHR-t678 than in KK-1 cells expressing the hFSHR-wt. KK-1 cells transiently transfected with the hFSHR-wt or hFSHR-t678 were incubated with 30 nM hFSH for 16 h at 37 °C. The cells were then washed with a neutral and an acidic buffer to remove the free and bound hFSH and then incubated without (open bars) or with 0.3 nM hFSH (solid bars) for 30 min prior to the measurement of intracellular cAMP. Each bar shows the mean ± S.E.M. of three to five independent transfections. The numbers above the bars of the down-regulated cells display the hFSH-induced cAMP response expressed as % of the response of the control cells expressing the same receptor. This was calculated after subtraction of the basal from the stimulated levels of cAMP in the control and down-regulated cells. The asterisk indicates a statistically significant difference (*t*-test, $P \leq 0.05$) between the two groups of down-regulated cells.

complex results in a greater degree of down-regulation of the cell surface receptors in cells expressing hFSHR-t678 when compared with cells expressing the hFSHR-wt (Krishnamurthy *et al.* 2003). A similar phenomenon is demonstrated herein (Fig. 4) in transiently transfected KK-1 cells expressing these two receptors at a similar density. KK-1 cells transiently expressing the hFSHR-wt and exposed to increasing concentrations of hFSH experienced a ~40% loss of cell surface receptors whereas KK-1 cells expressing hFSHR-t678 experienced a ~60% loss.

The more pronounced loss of receptors observed in cells expressing the hFSHR-t678 was accompanied by a more pronounced loss of cAMP responsiveness when the cells were exposed to a second challenge with hFSH (Fig. 5). The cAMP response of down-regulated cells expressing the hFSHR-wt was ~50% of the control cells whereas the cAMP response of down-regulated cells expressing the hFSHR-t678 was ~40% of the control cells. Again, it is worth noting that this loss of responsiveness was due to a differential decrease in the magnitude of the cAMP response in cells re-challenged with hFSH (compare the solid bars between the right- and left-hand panels in Fig. 5) rather than to a

differential changes in the basal levels of cAMP induced by the initial hFSH stimulation (compare the open bars between the right- and left-hand panels in Fig. 5). In fact, the basal levels of cAMP in the two groups of control and down-regulated cells were statistically indistinguishable.

Repeated attempts to measure an increase in estradiol levels in KK-1 cells transfected with the hFSHR-wt and incubated with hFSH and androstenedione were unsuccessful. Thus, the effects of hFSHR down-regulation on hFSH-induced steroid synthesis could not be ascertained.

Relationship between gonadotropin receptor density and cellular responses

The interpretation of many of the experiments described above is based on the assumption that changes in the cAMP or progesterone responses are caused by changes in receptor density rather than changes in their downstream effectors or the metabolic pathways leading to progesterone biosynthesis.

The changes in cAMP accumulation reported here (Figs 2 and 5) could in theory be attributed to changes in

Figure 6 The hCG-induced cAMP response is proportional to LHR density in MA-10 cells transfected with the hLHR-wt or hLHR-t682. MA-10 cells were transiently transfected with the indicated amounts of hLHR-wt or hLHR-t682 plasmids. ^{125}I -hCG binding (top panel) was measured during an overnight incubation at 4°C and the hCG-induced intracellular cAMP response (bottom panel) was measured during 30 min with 0.3 nM hFSH as described in Materials and methods. Each point shows the average $\pm$ S.E.M. of three to five independent transfections.

receptor density and/or changes in the functional properties, or the levels of G proteins or adenylyl cyclase. A number of experimental strategies using during the past three decades, however, have clearly documented that gonadotropin-induced changes in cAMP accumulation are not due to changes in the levels

or the functional properties of G proteins or adenylyl cyclase. When cells expressing the endogenous or transfected gonadotropin receptors are exposed to gonadotropins, the subsequent loss of the gonadotropin-induced cAMP response is in fact specific to the homologous hormone as documented by the finding that

Figure 7 The hFSH-induced cAMP response is proportional to FSHR density in KK-1 cells transfected with the hFSHR-wt or hFSHR-t678. KK-1 cells were transiently transfected with the indicated amounts of hFSHR-wt or hFSHR-t678 plasmids. ^{125}I -hFSH binding (top panel) was measured during an overnight incubation at 4°C and the hFSH-induced intracellular cAMP response (bottom panel) was measured during 30 min with 0.3 nM hFSH as described in Materials and methods. Each point shows the average $\pm$ S.E.M. of three to five independent transfections.

the cAMP responses of gonadotropin-treated cells to other stimuli such as cholera toxin or forskolin remain unchanged (Conti *et al.* 1977, Catt *et al.* 1980, Dufau *et al.* 1980, Darbon *et al.* 1984, Pereira *et al.* 1988, Hafez & Ascoli 1990, Sánchez-Yagüe *et al.* 1993, Ascoli 1996,

1997). To ensure that receptor density is indeed a limiting factor in cellular responsiveness, we transfected MA-10 or KK-1 cells with different amounts of the hLHR and hFSHR constructs used here and we tested their ability to respond to hCG or hFSH

respectively with an increase in cAMP accumulation. The experiments presented in Figs 6 and 7 show that there is a clear dependency of gonadotropin-induced cAMP responsiveness on receptor density.

With regards to the hCG-induced progesterone response in MA-10 cells it is already known that this response is limited not only by the density of LHR but also by the availability of cholesterol (Freeman & Ascoli 1981, 1982). Although the experiments presented here were done under conditions where cholesterol can become depleted (Fig. 3) this depletion should not affect the interpretation of the data presented because we have previously shown that the steroidogenic response of these cells to hCG is limited by the density of receptors even when an appropriate source of exogenous cholesterol is present (Freeman & Ascoli 1982). Lastly, when MA-10 cells are transfected with the hLHR (as done herein) there is a strong, positive correlation between the hCG-induced progesterone response and the density of receptors expressed (Hirakawa *et al.* 2002).

Therefore the hCG-induced loss of cAMP and progesterone responses detected in MA-10 cells (Figs 2 and 3) and the hFSH-induced loss of cAMP response detected in KK-1 cells (Fig. 5) are most likely due to the hCG- and hFSH-induced changes in the density of gonadotropin receptors (Figs 1 and 4).

Discussion

The potential involvement of the different fate of the internalized hormone receptor complexes and the differential loss of cell surface receptors on the initiation, termination or desensitization of cellular responses is not fully understood. With regards to the initiation of cellular responses, it appears that some signal transduction events generated by G protein-coupled receptors originate at the cell surface whereas others are generated from an endosomal compartment (Luttrell & Lefkowitz 2002, Sorkin & von Zastrow 2002). In the case of the mLHR, we showed a number of years ago that cAMP accumulation is a result of signals that are generated at the plasma membrane because this response stops upon removal of the surface-bound hCG from MA-10 cells even after a substantial proportion of the hormone receptor has been internalized (Segaloff & Ascoli 1981). Therefore, in this case, the internalization of the receptor-bound hormone appears to be involved in the termination of cAMP responses. Since cAMP accumulation is essential for steroid synthesis (Pereira *et al.* 1987, Wang & Ascoli 1990, Swinnen *et al.* 1991) and for the activation of the ERK1/2 pathway (Hirakawa & Ascoli 2003b), one can safely assume that the activation of these downstream pathways would also stop upon removal of the surface-bound hCG. This has been formally shown

only for steroid synthesis, however (Segaloff & Ascoli 1981).

Here we have examined the importance of the post-endocytotic fate of the internalized hLHR and hFSHR on the process of receptor down-regulation and the desensitization of gonadotropin responses using the wild-type and mutant forms of the hLHR and the hFSHR expressed in MA-10 and KK-1 cells respectively. The mutants of the hLHR and hFSHR used were chosen because they are routed mostly to a lysosomal degradation pathway whereas their wild-type counterparts are recycled back to the membrane. We have shown that agonist stimulation of cells expressing the mutant gonadotropin receptors results in a more pronounced loss of cell surface receptors and agonist responses than agonist stimulation of cells expressing the wild-type receptors. We conclude, therefore, that receptor recycling promotes the maintenance of gonadotropin receptors at the cell surface, thus preserving hormonal responsiveness even under continuous hormonal stimulation. Receptor degradation, on the other hand, promotes the loss of cell surface receptors and hormonal responsiveness under continuous hormonal stimulation.

Whereas the post-endocytotic fate of the FSHR from humans and rodents is the same (Krishnamurthy *et al.* 2003), the hLHR recycles more efficiently than the rodent LHR (Ascoli 1984, Kishi & Ascoli 2000, Kishi *et al.* 2001, Galet *et al.* 2003, 2004, Hirakawa *et al.* 2003). One must wonder then whether there is an evolutionary advantage to this change in the properties of the LHR. We speculate that the more efficient recycling of the hLHR is important in human physiology because it promotes the maintenance of hormonal responsiveness in the maternal luteal cells and the fetal Leydig cells during pregnancy when hCG levels are high.

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