

## Biological function of the LH receptor is associated with slow receptor rotational diffusion

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### Abstract

The biological activity of luteinizing hormone (LH) receptors can be affected by modifications to the receptor's amino acid sequence or by binding of hormone antagonists such as deglycosylated hCG. Here we have compared rotational diffusion of LH receptors capable of activating adenylate cyclase with that of non-functional hormone-occupied receptors at 4°C and 37°C using time-resolved phosphorescence anisotropy techniques. Binding of hCG to the rat wild-type receptor expressed on 293 cells (LHR-wt cells) or to the LH receptor on MA-10 cells produces functional receptors which exhibit rotational correlation times longer than 1000  $\mu$ s. However, modification of the LH receptor by substitution of Lys583  $\rightarrow$  Arg (LHR-K583R) results in a receptor that is non-functional and which has a significantly shorter rotational correlation time of  $130 \pm 12$   $\mu$ s following binding of hCG. When these receptors are treated with deglycosylated hCG, an inactive form of hCG, the rotational correlation times for the LH receptors on LHR-wt and MA-10 cells are also shorter, namely  $64 \pm 8$  and  $76 \pm 14$   $\mu$ s, respectively. Finally, a biologically active truncated form of the rat LH receptor expressed in 293 cells (LHR-t631) has slow rotational diffusion, greater than 1000  $\mu$ s, when occupied by hCG and a significantly shorter rotational correlation time of  $103 \pm 12$   $\mu$ s when occupied by deglycosylated hCG. The effects of rat LH binding to LH receptors on these various cell lines were similar to those of hCG although the magnitude of the changes in receptor rotational diffusion were less pronounced. We suggest that functional LH receptors are present in membrane complexes that exhibit slow rotational diffusion or are rotationally immobile. Shorter rotational correlation times for non-functional hormone-receptor complexes may reflect the absence of essential interactions between these complexes and other membrane proteins. © 2000 Elsevier Science B.V. All rights reserved.

**Keywords:** Luteinizing hormone receptor; Luteinizing hormone; Human chorionic gonadotropin; Time-resolved phosphorescence anisotropy; Rotational dynamics

Abbreviations: rLH, rat luteinizing hormone; hCG, human chorionic gonadotropin; degly-hCG, deglycosylated hCG; LHR-wt, 293 human embryonic kidney cells expressing the wild-type rat LH receptor; LHR-t631, 293 human embryonic kidney cells expressing the rat LH receptor truncated at residue 631; LHR-K583R, 293 cells expressing LH receptors containing a substitution of Lys581 with Arg; ErITC, erythrosin isothiocyanate; BSS, balanced salt solution; PBS, phosphate-buffered saline

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### 1. Introduction

The luteinizing hormone (LH) receptor is a member of the G protein-coupled receptor family. Although this receptor is a monomeric glycoprotein [1], its slow rotational diffusion in the plasma membrane suggests that the receptor may be present in large complexes. For example, the LH receptor appears rotationally immobile following binding of

hCG to receptors on ovine luteal cells [2] and on MA-10 cells [3] when examined by time-resolved phosphorescence anisotropy techniques. In contrast to the LH receptor, the Type I Fc $\epsilon$  receptor has rotational correlation times that are short, approximately 30  $\mu$ s, at 37°C [4]. This multi-subunit receptor has a total of seven membrane spanning regions and exists as a monomer until crosslinked by antigen [5].

The rotational diffusion of LH receptors also depends on the hormone bound to the receptor. Although LH receptors on rat and ovine luteal cells can bind hCG and LH, the receptor complex formed following binding of hCG has considerably slower rotational diffusion than does the complex formed following LH binding [2,6]. Despite apparent differences in complex size, the receptor complexes on ovine luteal cells [7] or Leydig tumor cells [8] following binding of LH and hCG are biologically functional and thus capable of activating cellular pathways leading to secretion of a steroid hormone product.

The goal of this project was to compare the rotational dynamics of LH receptors under conditions in which hormone binding to the receptor produced a biologically functional or non-functional receptor complex. Because the rotational correlation times of membrane proteins depend linearly on the in-membrane volume of the complex identified by the phosphorescent probe [9], we hypothesized that rotational diffusion measurements would be sensitive indicators of protein–protein associations that might be critical for biological function. Such function can be substantively altered by several strategies including deleting portions of the receptor, substituting amino acids within the receptor, or binding a non-functional ligand such as deglycosylated hCG to the receptor. Here we have examined the rotational diffusion of various forms of the LH receptor stably expressed in 293 cells. One mutant LH receptor, truncated at residue 631 in the cytoplasmic domain, exhibits biological activity in response to hCG binding, accompanied by delayed receptor desensitization [10]. A second form of the receptor contained a substitution of arginine for lysine at amino acid 583 (LHR-K583R) which lies at the juncture of the receptor's exo-loop 3 and a membrane spanning amino acid sequence [11]. Binding of hCG to this receptor

results in no cAMP elevation. We have also examined the rotational dynamics of non-functional hormone–receptor complexes formed by binding of chemically deglycosylated hCG [12] to wild-type LH receptors on both LHR-wt cells and MA-10 cells.

## 2. Materials and methods

### 2.1. Reagents and media

Dulbecco's modified Eagle's medium was purchased from Irvine Scientific, Santa Ana, CA. Gentamicin and geneticin were purchased from Gibco, Grand Island, NY. Hepes was purchased from Sigma Chemical Co., St. Louis, MO. Fetal bovine serum was purchased from Summit Biotechnology, Fort Collins, CO. Rat LH (rLH, NIH I-9) and hCG (CR-127) were obtained from the National Hormone and Pituitary Program, NIADDK, Baltimore, MD. Chemically deglycosylated hCG [12] was a gift from Dr. Henry Keutmann. Erythrosin isothiocyanate was purchased from Molecular Probes, Eugene, OR.

### 2.2. Cell culture

Dr. Tae Ji of the University of Wyoming Department of Molecular Biology generously provided 293 cells stably transfected with the wild-type LH receptor (LHR-wt) and 293 cells expressing an LH receptor with lysine 583 replaced by arginine (LHR-K583R; [11]). Human embryonic kidney cell lines stably transfected with a truncated form of the receptor ending at residue 631 (LHR-t631; [10]) were kindly provided by Dr. Deborah Segaloff of the University of Iowa Department of Physiology. Human kidney 293 cells were maintained in Dulbecco's modified Eagle's medium containing 50  $\mu$ g/ml gentamicin, 10% fetal bovine serum, and 10 mM Hepes, pH 7.4. Stably transfected 293 cells were maintained in the same medium supplemented with 700  $\mu$ g/ml geneticin. MA-10 cells were provided by Dr. Mario Ascoli of the University of Iowa Department of Pharmacology and were maintained in culture using procedures described previously [3]. Cell cultures were kept at 37°C in 5% CO<sub>2</sub> in air.

### 2.3. Preparation of ErITC-derivatized hormones

Hormones were derivatized with erythrosin isothiocyanate (ErITC) using a modification of methods described by Johnson and Holborow [13] that has been described in detail previously [2]. The molar ratios for rLH, hCG, or deglycosylated hCG derivatized with ErITC and the concentration of protein in solution were determined spectrophotometrically. The hormone preparations used in these experiments had 1.6 and 1.2 mol of ErITC per mol of rLH and hCG, respectively. Deglycosylated hCG had 1.4 mol of ErITC per mol of hormone. It has been previously shown that there is no effect of fluorophore conjugation on hormone biological activity [14]. Prior to use, all fluorophore-derivatized proteins were centrifuged at  $130\,000\times g$  for 5 min in a Beckman Airfuge (Beckman Instruments, Palo Alto, CA) to remove any protein aggregates which may have formed during storage at 4°C.

### 2.4. Labeling cells with erythrosin-derivatized hormones

Typically,  $10^7$  cells in 1 ml of balanced salt solution were labeled with ErITC-derivatized probe for each time-resolved phosphorescence anisotropy experiment. Cells were incubated in balanced salt solution containing 0.1%  $\text{NaN}_3$  at 37°C for 30 min to prevent hormone internalization [14] prior to addition of 1 nM ErITC-derivatized hormones for 1 h. Cells were then washed two times by centrifugation at  $300\times g$  for 3 min in balanced salt solution to remove any unbound ligand. To verify that there were no non-specific interactions of hormone with human kidney 293 cells, 293 cells that did not contain expression vectors for the LH receptors were treated with 1 nM ErITC-rLH. To determine whether binding of ErITC-derivatized hormones was specific, cells were preincubated with excess rLH or hCG prior to labeling with the respective ErITC-derivatized hormone in some experiments. In both cases, there was no phosphorescence signal detected using the time-resolved phosphorescence anisotropy apparatus described below.

### 2.5. Time-resolved phosphorescence anisotropy measurements

After labeling with ErITC-derivatized hormones, cells were deoxygenated for 15 min by purging with argon gas to eliminate phosphorescence quenching caused by  $\text{O}_2$ . The cell sample was then placed in a 5-mm Suprasil quartz cuvette (Helma Cells, Jamaica, NY) and inserted into a thermostated cuvette holder. Experiments were performed at 4°C and 37°C. The frequency-doubled 532 nm output of a Spectra-Physics DCR-11 Nd:YAG laser (Spectra-Physics, Albuquerque, NM) provided the excitation pulse for each experiment. The laser was operated at 10 Hz with a vertically polarized  $\text{TEM}_{00}$  output of 0.19 mJ and a beam  $1/e^2$  radius of 3.6 mm. Beam diameter was restricted to 2.5 mm at the sample. Phosphorescence emission from the sample was collected 90° to the excitation axis and isolated with a 1-cm pathlength of 1 M  $\text{Na}_2\text{Cr}_2\text{O}_7$  solution, a KV 550 color filter (Schott Glass Technologies, Duryea, PA) and a 3-mm thick RG 665 filter. A rotating polarizer was placed in front of the photomultiplier tube in order to observe the intensity of phosphorescence with polarization parallel and perpendicular to the exciting pulse. The phosphorescence signal was collected by a thermionically cooled EMI 9816A photomultiplier tube. A fast gating circuit was used to turn the photomultiplier tube off during the high-power Nd:YAG pulses [15]. The output signal from the photomultiplier tube was amplified by a Tektronix 476 oscilloscope. The oscilloscope was further amplified by a 35-MHz bandwidth buffer amplifier and fed to a Nicolet 12/70 signal averager equipped with a 20-MHz analog-to-digital converter. Phosphorescence decay traces from 4096 laser pulses were averaged for each polarizer orientation, the channel width being 0.5  $\mu\text{s}$ . After data acquisition was complete, the data were downloaded into a 80486 microcomputer for data analysis and storage, as has been previously described [3]. Total phosphorescence emission was analyzed according to a multi-exponential decay model and an apparent half-time for phosphorescence decay calculated. Analysis of anisotropy decay yielded the initial anisotropy value  $r_0$ , the limiting anisotropy value  $r_\infty$ , and the rotational correlation time  $\phi$  as well as the statistical uncertainties in these quantities [16].

### 3. Results

#### 3.1. Phosphorescence lifetimes reflect temperature and type of protein binding the phosphor

Phosphorescence decay from xanthine dyes like erythrosin is typically complex, especially when these dyes are conjugated to proteins. Analyses of experimental data typically involves use of a linear combination of exponential decays and three exponentials of adjustable time constants and fractional amplitudes [4]. It is, however, difficult to compare decay kinetics among samples analyzed in this way since both time constants and amplitudes change between samples. An alternate strategy is to fit intensity decay to a *fixed* set of time constants which span the full range of decay phenomena measurable, from the fastest to the slowest. The result of such a fit is effectively a histogram of the distribution of time constants comprising the decay. This is the strategy we have taken with data in this study (Table 1). Using six *fixed* decay times evenly spaced from 3 to 1000  $\mu$ s, we obtained fits indistinguishable from those ob-

tained with three *adjustable* decay times and accrued the benefit of being able to see from what part of the decay constant distribution specific lifetime effects arise. While the apparent half-times for phosphorescence decay from cell-bound hormone conjugates fell within the quite narrow range of 28–86  $\mu$ s, each analyzed decay trace contained components covering the entire 3–1000  $\mu$ s range.

The lifetimes seemed most strongly affected by the nature of the ligand bound. For any specific cell type and temperature, the average half-time for phosphorescence decay increased in the order LH < degly-hCG < hCG. Thus, for erythrosin-ligands bound to LHR-wt at 4°C, the apparent half-time was 39  $\mu$ s for LH, 61  $\mu$ s for degly-hCG and 86  $\mu$ s for hCG. Decay half-times decreased with increasing temperature for all samples examined, including those shown in Table 1. Half-times decreased from 2.4-fold (hCG) to 1.4-fold (LH) over this 4–37°C range. For each ligand, the center of mass of the decay histogram shifted to faster decays at 37°C, but no gross changes in the histogram shape were apparent.

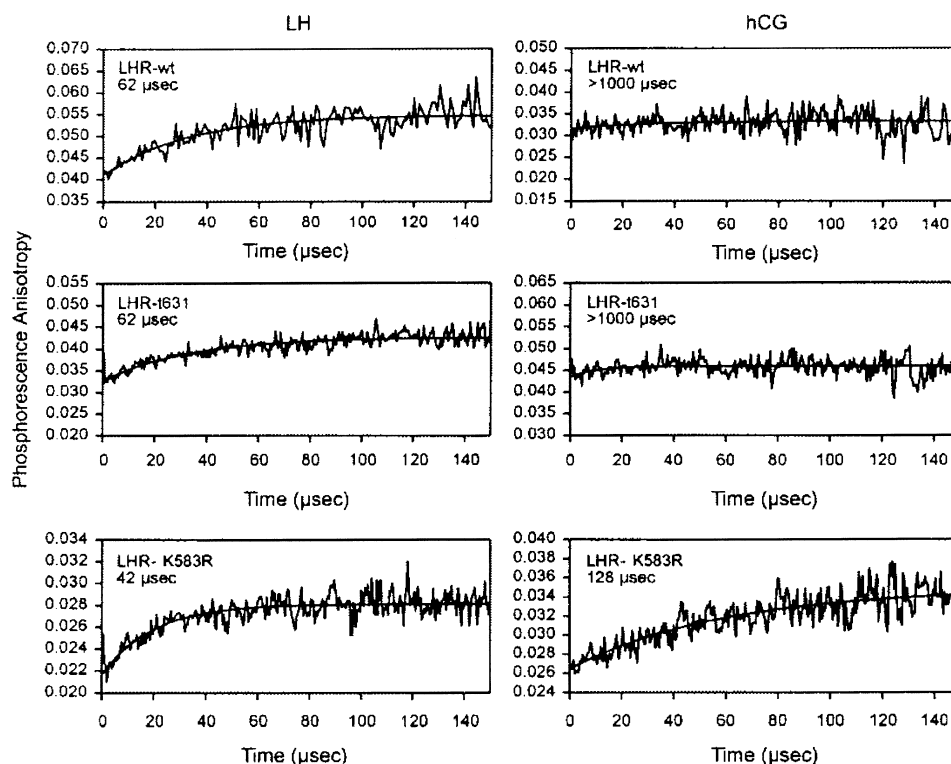


Fig. 1. Comparison of phosphorescence anisotropy decay at 37°C for either ErITC-rLH or ErITC-hCG bound by LH receptors on LHR-wt, LHR-t631 and LHR-K583R cells.

Table 1

Representative phosphorescence intensity decay parameters for hormone erythrosin conjugates to LH receptors on LHR-wt cells<sup>a</sup>

Ligand	Temp. (°C)	$t_{1/2}$ (μs)	Fractional amplitudes (%)					
			3 μs	10 μs	30 μs	100 μs	300 μs	1000 μs
hCG	4	86	0	12	11	32	32	13
	37	36	0	18	32	25	19	6
LH	4	39	5	15	27	18	30	4
	37	28	7	20	29	19	23	2
degly-hCG	4	61	0	21	12	30	21	16
	37	32	0	23	29	22	25	1

<sup>a</sup>Tabulated quantities are representative fits of phosphorescence intensity to the indicated six exponentials with results presented as percents of the total amplitude. The indicated half-times were calculated from the resulting fitted curve.

### 3.2. Functional receptor–ligand complexes exhibit slow rotational diffusion at 37°C

We compared the rotational motions of biologically functional LH receptor–ligand complexes with those of non-functional complexes. Table 2 and Fig. 1 show the rotational parameters of the various receptor–ligand combinations at 37°C. Due to structural differences between LH and hCG which affect their molecular motions, comparisons were best made for a specific ligand binding different forms of the LH receptor. Most, if not all, hCG-occupied LH receptors on LHR-wt, MA-10 and LHR-t631 cells were rotationally immobile on the timescale of our experiments as indicated by differences between  $r_0$  and  $r_\infty$  of 10% or less (Table 2) and all these

systems exhibit biological activity. By contrast, the non-functional LHR-K583R receptors binding hCG were mobile with an observed rotational correlation time of  $130 \pm 12$  μs. Differences were similar, if somewhat less pronounced, among receptors binding LH. LH-Occupied receptors on LHR-wt and LHR-t631 cells exhibited rotational correlation times of  $62 \pm 7$  and  $61 \pm 11$  μs, respectively. Non-functional receptors on LHR-K583R cells exhibited the more rapid anisotropy decay time of  $42 \pm 6$  μs.

### 3.3. Differences in rotational kinetics were less pronounced at 4°C

As shown in Table 3 hCG-occupied receptors on each cell type were rotationally mobile at 4°C. Func-

Table 2

Time-resolved anisotropy decay data for LH receptors at 37°C<sup>a</sup>

Ligand	Cell line	$r_0^b$	$r_\infty^b$	$\Delta r/r_\infty^b$	$\phi^c$ (μs)	$t_{1/2}$ (μs)	$n$
hCG	LHR-wt	$0.035 \pm 0.001$	$0.039 \pm 0.001$	0.10	> 1000	36	5
	MA-10	$0.480 \pm 0.001$	$0.048 \pm 0.001$	0.0	> 1000	ND	16
	LHR-t631	$0.037 \pm 0.006$	$0.040 \pm 0.005$	0.08	> 1000	33	8
	LH-K583R	$0.034 \pm 0.008$	$0.046 \pm 0.010$	0.26	$130 \pm 12$	39	9
LH	LHR-wt	$0.043 \pm 0.002$	$0.056 \pm 0.002$	0.23	$62 \pm 7$	27	4
	LHR-t631	$0.039 \pm 0.004$	$0.049 \pm 0.005$	0.20	$61 \pm 11$	28	7
	LH-K583R	$0.037 \pm 0.017$	$0.047 \pm 0.022$	0.21	$42 \pm 6$	29	7
degly-hCG	LHR-wt	$0.056 \pm 0.003$	$0.067 \pm 0.003$	0.16	$63 \pm 8$	33	8
	MA-10	$0.040 \pm 0.004$	$0.047 \pm 0.005$	0.15	$76 \pm 14$	33	10
	LHR-t631	$0.052 \pm 0.001$	$0.061 \pm 0.002$	0.15	$103 \pm 12$	30	5

<sup>a</sup>Cells were labeled with 1 nM ErITC-rLH, ErITC-hCG or ErITC-deglycosylated hCG as described in Section 2. For each sample, 2048 measurements of phosphorescence decay were averaged to obtain the  $I_{||}$  and  $I_{\perp}$  traces from which anisotropy was calculated.

<sup>b</sup> $r_0$  is mean and S.D. of the initial anisotropy and  $r_\infty$  is the mean and S.D. of the final anisotropy. The ratio  $\Delta r/r_\infty = |r_\infty - r_0|/r_\infty$  provides a measure of the rotational freedom of the LH receptor population being examined. Where values for  $r_\infty$  and  $r_0$  differ by less than 10%, the rotational diffusion is slower than can be measured on the time scale of our experiments and, as such, is greater than 1000 μs.

<sup>c</sup>The rotational correlation times ( $\phi$ ) are the mean and S.D. of measurements on  $n$  samples examined at each temperature.

tional LHR-wt and LHR-t631 receptors exhibited rotational correlation times ranging of 125 and 93  $\mu$ s, respectively, while that of the non-functional LHR-K583R receptors was 113  $\mu$ s. However, small differences between the initial and final anisotropies,  $r_0$  and  $r_\infty$ , resulting in  $\Delta r/r_\infty$  ratios of 0.11–0.16 for hCG treated cells, suggest that the number of mobile receptors was not large. Among LH-binding receptors, the non-functional LHR-K583R exhibited a more rapid rotational correlation time of  $80 \pm 13$   $\mu$ s than did either of the functional systems, namely LHR-wt ( $93 \pm 5$   $\mu$ s) or LHR-t631 ( $89 \pm 10$   $\mu$ s). However, these differences were not significant statistically.

### 3.4. hCG binding receptors typically exhibited more restricted rotational motion than receptors binding other ligands

At both 4°C and at 37°C, hCG-binding receptors as a group exhibited slower rotational diffusion and smaller  $\Delta r/r_\infty$  ratios than receptors binding other ligands. For example, at 37°C, rotational correlation times for receptor-bound hCG ranged from  $>1000$   $\mu$ s to 130  $\mu$ s while, for other ligands, values ranged from 42 to 103  $\mu$ s. For every individual receptor type at either temperature, hCG-occupied receptors had rotational correlation times that were longer than those for LH-occupied receptors. This is reminiscent of phosphorescence intensity decay data where recep-

tor-bound hCG invariably exhibits slower decay than other ligand. In addition, more LH-occupied receptors were mobile, as indicated by larger  $\Delta r/r_\infty$  ratios, than were hCG-occupied receptors. For example, for LHR-wt receptors at 37°C,  $\Delta r/r_\infty$  was 0.23 for LH compared with 0.10 for hCG.

### 3.5. The non-functional ligand deglycosylated hCG affects receptor rotation differently from hCG

To determine whether binding of a non-functional ligand affected the rotational motions of LH receptors, we examined the diffusion of LH receptors on LHR-wt and LHR-t631 cells and on the MA-10 murine Leydig tumor cell line where binding of deglycosylated hCG results in slower internalization of LH receptors [17] and little or no receptor phosphorylation. As shown in Tables 2 and 3, LH receptors occupied by deglycosylated hCG were rotationally mobile at 4°C and 37°C. At 37°C, the  $\Delta r/r_\infty$  ratios were 0.10, 0.16 and 0.23 for LHR-wt receptors binding hCG, degly-hCG and LH, respectively. Thus the relative number of mobile receptors occupied by deglycosylated hCG was intermediate between that of hCG- and LH-occupied receptors. More importantly, LH receptors occupied by deglycosylated hCG had significantly shorter rotational diffusion times (63–103  $\mu$ s) than did the same receptors occupied by hCG on each cell type examined.

Table 3  
Time-resolved anisotropy decay data for LH receptors at 4°C<sup>a</sup>

Ligand	Cell line	$r_0^b$	$r_\infty^b$	$\Delta r/r_\infty^b$	$\phi^c$ ( $\mu$ s)	$t_{1/2}$ ( $\mu$ s)	$n$
hCG	LHR-wt	$0.046 \pm 0.003$	$0.055 \pm 0.004$	0.16	$125 \pm 16$	83	7
	LHR-t631	$0.041 \pm 0.004$	$0.046 \pm 0.004$	0.11	$93 \pm 21$	61	7
	LH-K583R	$0.039 \pm 0.005$	$0.046 \pm 0.006$	0.15	$113 \pm 16$	71	9
LH	LHR-wt	$0.032 \pm 0.003$	$0.042 \pm 0.005$	0.25	$93 \pm 5$	41	7
	LHR-t631	$0.036 \pm 0.001$	$0.048 \pm 0.002$	0.25	$89 \pm 10$	46	6
	LH-K583R	$0.033 \pm 0.004$	$0.042 \pm 0.005$	0.21	$80 \pm 13$	43	9
degly-hCG	LHR-wt	$0.049 \pm 0.003$	$0.058 \pm 0.005$	0.16	$57 \pm 14$	33	7
	LHR-t631	$0.049 \pm 0.007$	$0.056 \pm 0.008$	0.12	$52 \pm 10$	51	8
	MA-10	$0.040 \pm 0.007$	$0.046 \pm 0.008$	0.13	$73 \pm 15$	60	9

<sup>a</sup>Cells were labeled with 1 nM ErITC-rLH, ErITC-hCG or ErITC-hCG as described in Section 2. For each sample, 2048 measurements of phosphorescence decay were averaged to obtain the  $I_{||}$  and  $I_{\perp}$  traces from which anisotropy was calculated.

<sup>b</sup> $r_0$  is the mean and S.D. of the initial anisotropy and  $r_\infty$  is the mean and S.D. of the final anisotropy. The ratio  $\Delta r/r_\infty = |r_\infty - r_0|/r_\infty$  provides a measure of the rotational freedom of the LH receptor population being examined.

<sup>c</sup>The rotational correlation times  $\phi$  are the mean and S.D. of measurements on  $n$  samples examined at each temperature.

#### 4. Discussion

The rotational dynamics of hormones bound to LH receptors on LHR-wt cells are similar to those of hormone-occupied LH receptors on ovine luteal cells [2] and to LH receptors on MA-10 murine Leydig tumor cells [3]. LH-occupied receptors on LHR-wt cells exhibit faster rotational diffusion at 37°C than at 4°C, a temperature dependence that, in all likelihood reflects the 2–3-fold change in membrane viscosity over this temperature range. Similarly, phosphorescence decay accelerates from 1.4-fold at 4°C to 2.6-fold at 37°C. This may reflect hormone conformational fluctuations causing collisions of excited state dye molecules with other membrane structures. Alternatively, the temperature coefficient may simply reflect faster diffusion of residual oxygen or other quenching solution species. Finally, rotational diffusion of LH receptors on LHR-wt cells at 37°C depends on whether LH or hCG is bound to the receptor as is the case for LH receptors on sheep luteal cells [2] and rat luteal cells [6].

At 37°C, comparison of receptors occupied either by LH or by hCG showed significant differences in rotational correlation times depending on whether the receptors were functional or non-functional. Modified LH receptors expressed on LHR-K583R cells exhibited shorter rotational correlation times following binding of rat LH or hCG than did functional receptors binding the same ligand. For LH-occupied receptors, the values for the ratio of  $\Delta r/r_\infty$  calculated from the initial and final anisotropies were comparable and this suggests that the relative number of mobile receptors is similar. The difference between hCG-occupied receptors on LHR-K583R cells and functional LH receptors is more striking. In addition to being rotationally mobile, considerably more receptors on LHR-K583R cells exhibit rotational diffusion.

Nonetheless, at 37°C rotational correlation times for the hCG-occupied receptor on LHR-K583R cells were longer,  $130 \pm 12 \mu\text{s}$ , than those of LH-occupied receptors on the three cell lines examined. Because this is the case, a proposed correlation between slow receptor rotational diffusion and receptor function involves an assumption that LH and hCG are structurally different and that these structural differences affect the size of the resulting receptor-containing

complex. This is arguably a reasonable assumption. The rotational diffusion of LH-occupied receptors are generally faster than those of hCG-occupied receptors where glycosylation of hCG is responsible, in part, for slowing receptor rotational diffusion. Binding of deglycosylated hCG to LH receptors on LHR-wt cells resulted in a 62- $\mu\text{s}$  rotational correlation time which was shorter than that of intact hCG and comparable to the rotational correlation time for receptors occupied by rLH. The effects of hCG deglycosylation on the lateral dynamics of LH receptors have been noted previously. The LH receptor on sheep luteal cells diffuses laterally following binding of deglycosylated hCG [14] but is laterally immobile [14], as well as rotationally immobile [2], following binding of hCG. Although a detailed mechanism is not known, the notion that carbohydrates on hCG bind membrane lectins has been proposed by Calvo and Ryan [18] and, indeed, anchoring of these membrane lectins within the membrane bilayer by the cytoskeleton or by extensive interactions with other membrane structures would account for slower lateral and rotational diffusion of the LH receptor following hCG binding. However, Bahl and coworkers have shown that oligosaccharides from hCG inhibit hormone binding to the receptor and, as a result, suggest that carbohydrates on the hormone interact directly with the receptor [19].

If rotational correlation times reflect the molecular mass of a protein complex in the membrane, longer rotational correlation times for the functional hormone–receptor complexes may be due to formation of larger complexes. The molecular components within these complexes have not been identified. The presence of LH receptor dimers or oligomers has been suggested by electron microscopy [20] as well as by positive values for energy transfer between LH receptors on luteal tissue from pig [21] and sheep [22]. In addition, other non-receptor proteins almost certainly interact or lie within close proximity of the LH receptor. This appears to be the case for LH receptors on plasma membrane preparations from bovine corpora lutea, where photoproximity labeling shows a number of as-yet unidentified proteins proximal to the hormone-binding LH receptor [23]. Thus, it seems possible that the size and composition of complexes containing non-functional LH receptors versus functional receptors are different.

Despite observed differences in the rotational correlation times for various combinations of hormone and receptor, the relative numbers of rotationally mobile receptors at 37°C was consistently small. Values for the ratio of  $\Delta r/r_\infty$  presented Table 2 ranged from 0.02 to 0.26. In contrast, the Fc receptor for IgE on rat basophilic leukemia cells, which is believed to be monomeric, has a  $\Delta r/r_\infty$  ratio of about 0.5 [24]. Thus differences in rotational correlation times for, as an example, hCG-occupied receptors on LHR-wt and LHR-K583R cells, reflects the motions of a small subset of the otherwise rotationally immobile receptor population. Whether small differences in the number of rotationally mobile receptors affects the overall biological activity of the receptor is not known. One can hypothesize that when binding of hormone to receptor produces a functional complex, some receptors move from an environment in which they are rotationally mobile into an environment, such as membrane rafts [25], where the receptor signals effectively and is rotationally immobile. Although there has been no direct demonstration of LH receptors within membrane rafts, the LH receptor may partition into more than one membrane environment. Bramley and Ryan [26] have shown that sucrose gradient fractionation of corpus luteum membranes results in two LH receptor-containing fractions, only one of which has adenylate cyclase activity.

Rising anisotropies for the LH- and hCG-occupied receptors on each of the cell types examined are similar to those seen for LH receptor on murine Leydig cells [27], ovine luteal cells [2] and MA-10 cells [3], as well as for epidermal growth factor receptor on plasma membrane vesicles shed from A-431 cells [28]. The conditions that may result in rising anisotropies have been discussed by Jovin and coworkers [9,29] who also cite other instances where rising anisotropies have been observed. A rising anisotropy is exhibited by a chromophore whose transition dipoles assume suitable orientations with respect to the axis about which it rotates. In such a case either or both of the exponential decay terms in the anisotropy decay expression for a uniaxial rotator can assume a negative amplitude, causing an anisotropy function which decays *upward* to a positive limiting value.

The apparent rotational immobility of receptor-

bound hCG needs to be considered in light of such photophysics of anisotropy decay. It is possible for a single chromophore to be bound to a rotating ligand with precisely the correct orientation so as to exhibit no anisotropy decay. Alternatively, two or more properly oriented chromophores can cancel each other's contributions to anisotropy decay kinetics. In either situation no time-dependent anisotropy decay would be observed from a receptor-bound ligand despite its undergoing well-defined rotational depolarization processes. However, the photophysical and structural conditions required for such a situation seem almost impossibly restrictive. Moreover, our direct photobleaching recovery measurements show that more receptor-bound hCG is laterally immobile on a variety of cell types than is receptor-bound LH which exhibits more typical lateral diffusion coefficients [14]. We thus feel that the fixed anisotropies observed for receptor-bound hCG are best interpreted in terms of receptor immobilization.

Taken together, these results suggest first that there are structural differences between the complexes formed by the LH and hCG on these cell lines and that the non-functional LH receptor occupied by hCG or the functional LH receptors occupied by deglycosylated hCG have substantive differences in their interactions within the plasma membrane.

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