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Differential Responses of an Invariant Region in the Ectodomain of Three Glycoprotein Hormone Receptors to Mutagenesis and Assay Conditions

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The glycoprotein hormone receptors—luteinizing hormone receptor (LHR), follicle-stimulating hormone receptor (FSHR), and thyroid-stimulating hormone receptor (TSHR)—are G-protein-coupled receptors with an invariant 10-amino acid residue sequence in the ectodomain proximal to transmembrane helix 1. A Glu-Asp, located at the midpoint of this conserved sequence, has been suggested to be important in ligand-mediated signaling of LHR and/or receptor expression or stability, but not binding. One goal of this study was to expand the studies on LHR and determine whether the invariant Glu and Asp residues were functional in FSHR and TSHR as well. Another goal was to investigate systematically the role of ionic strength, particularly Na^+, which appears to have enigmatic functions in the three receptors regarding ligand binding and receptor activation, and to ascertain whether any of the purported effects of Na^+ could involve the conserved pair of acidic side chains in the ectodomain. COS-7 cells were transiently transfected with cDNAs to the wild-type (WT) receptor (rat) and identical single and double mutants of each (Glu→Ala, Asp; Asp→Ala, Glu; and Glu-Asp→Asp-Glu), followed by characterization of cognate ligand binding and signaling (basal and hormone mediated) in two commonly used buffer systems: Waymouth's medium, containing a near-physiologic concentration of Na^+ (132 m*M*); a low ionic strength buffer with a 1 m*M* concentration of Na^+. The three receptors exhibited differential responses to mutagenesis and the two buffers. Notably, a comparison of basal cyclic adenosine monophosphate (cAMP) production showed that the buffer of lower ionic strength resulted in increased basal cAMP production in WT TSHR but not LHR and FSHR; that the maximal ligand-mediated cAMP production was greatest in the buffer of higher ionic strength for the three WT receptors; that functionality of the conserved Glu and Asp residues in ligand-mediated signaling was buffer dependent in LHR, whereas it did not appear to be particularly important in FSHR and TSHR signaling; and that apparent ligand binding in WT and mutant TSHRs seemed to be particularly diminished in the buffer of higher ionic strength. These results demonstrate that identical amino acid residues in homologous receptors can exhibit distinct functions; moreover, the role of ionic strength (Na^+) on signaling differs in the three receptors.

Key Words: G-protein-coupled receptors; signaling; cyclic adenosine monophosphate; adenylyl cyclase; forskolin.

Introduction

The receptors for luteinizing hormone (LH) and human chorionic gonadotropin (hCG) (LH receptor [LHR]), follicle-stimulating hormone (FSHR), and thyroid-stimulating hormone (TSHR) belong to the subfamily of glycoprotein hormone receptors within the G-protein-coupled receptor family. Each of the glycoprotein hormone receptors contains a relatively large N-terminal ectodomain responsible for high-affinity binding of cognate ligand(s), a bundle of seven membrane-spanning helices, three intracellular and three extracellular loops, and a short C-terminal tail comprising the endodomain. Within a given species each receptor contains an invariant sequence of 10 amino acid residues, FNPCEDIMGY, in the C-terminal portion of the ectodomain near transmembrane helix 1 (Fig. 1). Earlier studies, based on engineered mutations of LHR, suggested an important role of the invariant Glu-332 and Asp-333 in ligand-mediated signaling but not ligand binding *(1,2)*. Confirming the importance of this region in LHR, three siblings were reported who had homozygous mutations in their LHR resulting in a Glu→Lys replacement in the invariant ectodomain sequence *(3)*. This mutation, shown earlier to interfere with ligand-mediated receptor activation and/or reduce receptor expression or stability *(1,2)*, resulted in male pseudohermaphroditism and primary amenorrhea in 46XY and 46XX individuals, respectively *(3)*. This conserved region was considered to be part of a conformational switch relaying infor-

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Fig. 1. Partial amino acid sequences of LHR, FSHR, and TSHR ectodomains proximal to transmembrane helix 1. The location of the invariant 10-amino acid residue sequence is shaded, and the ED sequence studied herein is in bold.

mation of ligand contact with the ectodomain to the transmembrane portion of the receptor resulting in receptor activation *(1,2)*.

Unlike the gonadotropin receptors, LHR and FSHR, which exhibit minimal constitutive activity, TSHR displays some degree of constitutive activity in the absence of ligand *(4–6)*. Indeed, previous findings demonstrated differential sensitivity of LHR and TSHR to Na^+, suggesting that under normal physiologic conditions ionic interactions play a key role in maintaining LHR primarily in the inactive state and the constitutive activity of TSHR at a relatively low level; moreover, the maximal production of cyclic adenosine monophosphate (cAMP) at saturating concentrations of cognate hormone was greater in the presence of Na^+ for both LHR and TSHR *(6)*. Although one study found a decrease in the rate of association of hCG binding to LHR in the presence of Na^+ *(7)*, other studies found no effect of Na^+ on the binding affinity of hCG to LHR *(8,9)*, but there was a significant reduction in the binding affinity of ovine LH *(8)*. It has also been shown that Na^+ and other cations decrease the binding of TSH and FSH to their respective receptors *(10–12)*.

The aims of the present study were to elucidate the contribution of the invariant Glu and Asp residues in ligand-mediated signaling for the other members of the glycoprotein hormone receptor subfamily, FSHR and TSHR; and to conduct a comparative functional study under two commonly used assay conditions with particular emphasis on the role of Na^+ and ionic strength.

Results

Since other studies have shown differential sensitivity of the three glycoprotein hormone receptors to the concentration of Na^+ *(6,11)*, the binding and signaling experiments were performed in two different, but commonly used buffer systems: Waymouth's medium (WM), in which (Na^+) = 134 m*M* (isotonic [NaCl] buffer); and binding buffer (BB), in which (Na^+) = 1 m*M* in the presence of 278 m*M* sucrose (isotonic [sucrose] buffer). A comparative study of functionality of Glu and Asp in the invariant region, as well as the effect(s) of Na^+, was conducted by preparing and characterizing a series of identical single and double mutants of the three receptors: Glu→Ala, Asp; Asp→Ala, Glu; and Glu-Asp→Asp-Glu.

Figure 2 shows the results of specific binding of cognate ligand and maximal ligand-mediated cAMP production in the two buffers for the three receptors. Figure 3 shows representative competitive binding and cAMP dose-response curves for each of the wild-type (WT) and double mutant receptors. A complete summary of the results for the three receptors in the two buffers is provided in Tables 1 (LHR), 2 (FSHR), and 3 (TSHR), and the findings are presented separately for each of the receptors.

LHR

Several features emerge from analysis of the results in Figs. 2A and 3A and Table 1. The expression levels of the mutants were less than that of WT LHR, and the Ala replacements were expressed at lower levels than the E→D and D→E replacements. In BB, the IC_{50} and EC_{50} values of WT and mutant LHRs were similar (i.e., 0.06–0.09 n*M*), resulting in an IC_{50}/EC_{50} ratio of 1.0 ± 0.3. In WM, the IC_{50}s were greater than the corresponding values in BB for WT LHR and each mutant. The EC_{50} of WT LHR in WM was similar to that in BB; however, the dose-response curves for the LHR mutants were too shallow to permit accurate measurement of EC_{50} values. Basal cAMP production was the same for WT LHR and each mutant in BB and in WM. By contrast, the maximal cAMP production of WT LHR in response to hCG was greater in WM than in BB, but that for each mutant was less in WM than in BB. While the expression levels of the single mutants in BB exhibited about two- threefold differences, the maximal cAMP levels were remarkably constant. Likewise, a threefold difference in expression levels of the single mutants assayed in WM resulted in maximal hCG-mediated cAMP differences of less than twofold. In both BB and WM, the maximal production of cAMP in response to hCG for the reversal mutant, E332D/D333E, was significantly less than the values for WT LHR and the single mutants. This reduction cannot be attributed to expression levels since receptor density was comparable with or greater than that of the single mutants.

FSHR

The results in Figs. 2B and 3B and Table 2 show that the FSHR mutants, like those of LHR, were expressed at lower levels than WT FSHR. When assayed in BB, the IC_{50}s and EC_{50}s were similar, varying between about 0.2 and 1.1 n*M*, and the IC_{50}/EC_{50} ratios differed by twofold at most. In WM, the IC_{50} and EC_{50} values were greater than when determined in BB, and the IC_{50}/EC_{50} ratios varied as much as threefold. For the E339A and double mutants, cAMP responses were too minimal to permit meaningful determinations of EC_{50} values. Basal cAMP production was the same in BB and in WM for WT FSHR and the mutants. As was found for WT LHR, the maximal FSH-mediated cAMP production of WT FSHR was greater in WM than in BB, but there were no consistent patterns of the maximal levels of cAMP of the mutants in WM compared with BB, some being reduced

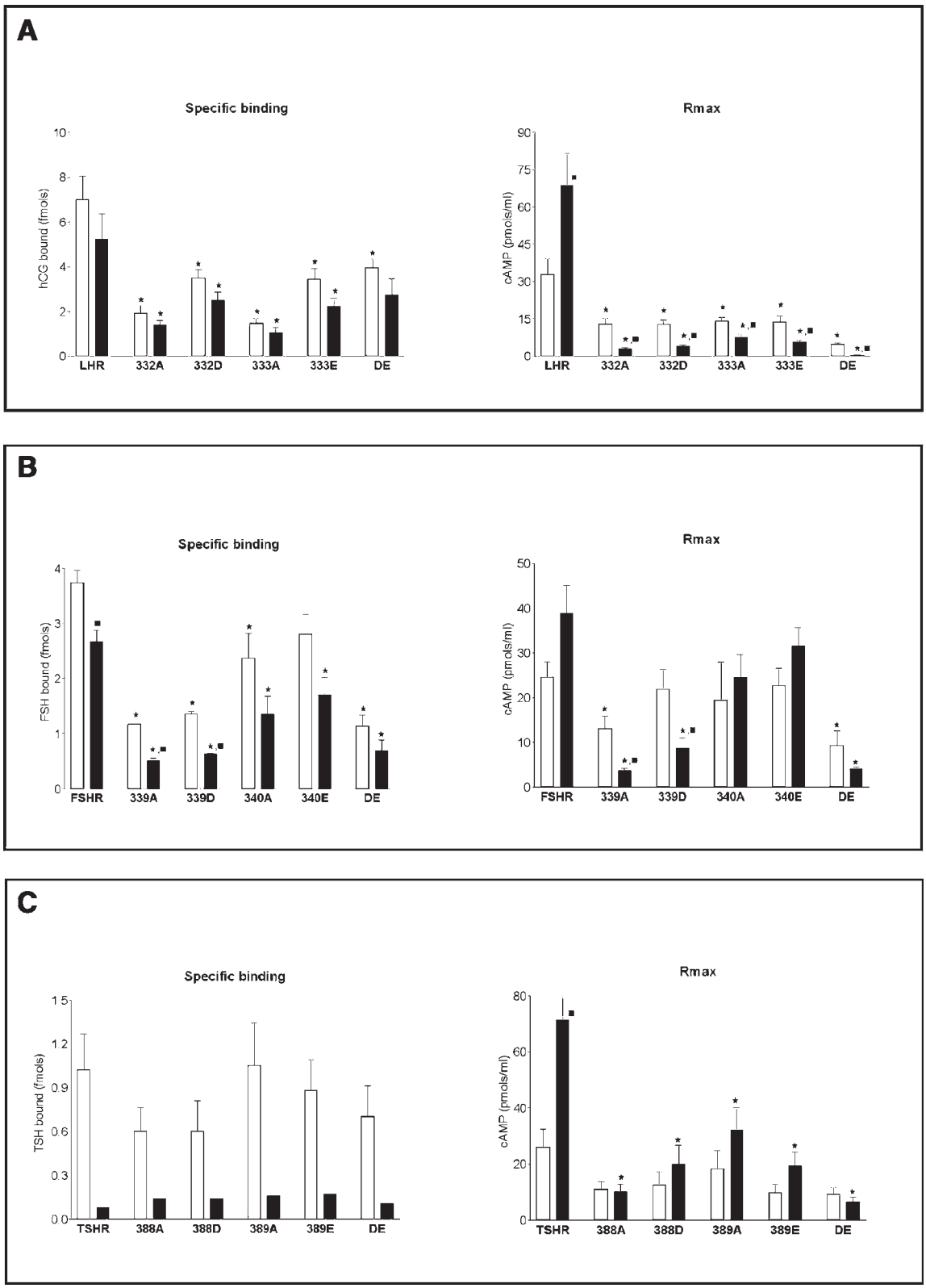

Fig. 2. B_o and maximal cAMP values for the three WT receptors—(**A**) LHR, (**B**) FSHR, and (**C**) TSHR—and for identical single and double mutants of Glu and Asp in invariant region of ectodomain, with measurements in two buffers, BB (□) and WM (■). In all cases, B_o values reflect apparent expression and were determined from specific binding measurements using ^{125}I-cognate hormone (6-h incubation at 37°C); maximal cAMP levels were determined following a 30-min incubation (37ºC) with a saturating dose of cognate hormone (100 ng/mL) in the presence of 0.8 m*M* IBMX. R_{max} is the maximal hormone-mediated cAMP production minus the basal level. ★, Values significantly different from WT receptor; ■, values significantly different in the two buffers. A statistical comparison of the specific binding of WT and TSHR mutants in the two buffers was not done since binding in WM was very low and was performed only once for comparison.

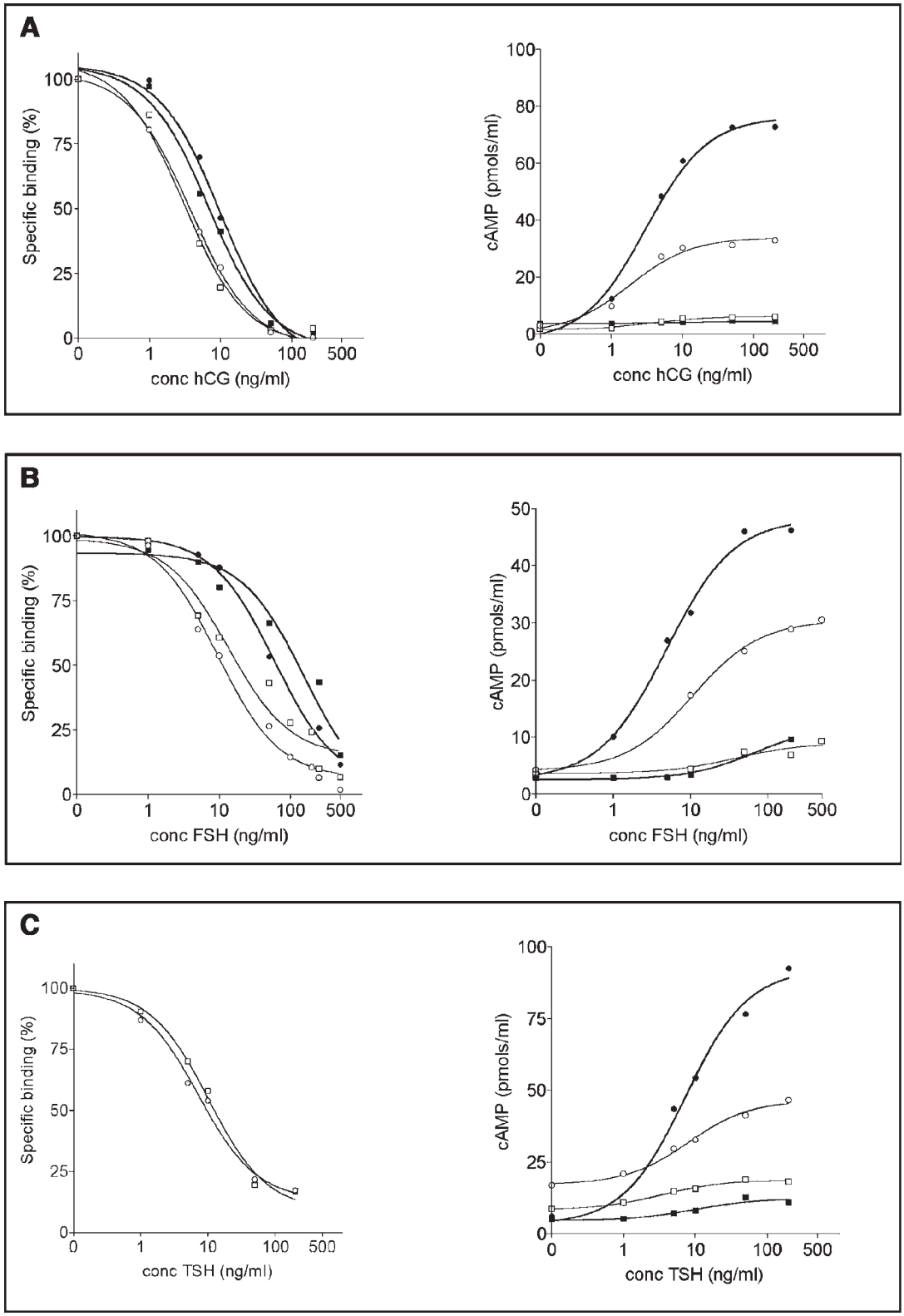

Fig. 3. Representative competitive binding and ligand-mediated signaling (cAMP) responses of cells expressing WT (circles) and double mutants (DE, i.e., E→D/D→E) (squares) in BB (open symbols) and WM (solid symbols): (**A**) LHR, (**B**) FSHR, and (**C**) TSHR). Binding studies were performed with ^{125}I-cognate hormone and unlabeled hormone (6 h, 37ºC), and cAMP responses were determined after a 30-min incubation (37ºC) with unlabeled hormone (100 ng/mL) in the presence of 0.8 m*M* IBMX. The data shown are from a representative experiment; means ± SEM of binding and signaling parameters from replicate transfections are given in Tables 1–3 for the three receptors.

Table 1
Binding and Signaling Characteristics of LHR in Two Buffers[a]

LHR	B_o (fmol)	IC_{50} (n*M*)	bcAMP (pmol/mL)	EC_{50} (n*M*)	mcAMP (pmol/mL)
BB:					
wt	7.0 ± 1.1 (6)	0.07 ± 0.005 (3)	2.8 ± 0.4 (6)	0.06 ± 0.008 (3)	35.7 ± 6.5 (6)
E332A	1.9 ± 0.3 (7)	0.07 ± 0.01 (3)	2.9 ± 0.5 (7)	0.06 ± 0.006 (3)	15.9 ± 2.4 (7)
E332D	3.5 ± 0.4 (7)	0.09 ± 0.03 (3)	2.4 ± 0.4 (7)	0.07 ± 0.005 (3)	15.1 ± 1.8 (7)
D333A	1.5 ± 0.2 (7)	0.06 ± 0.01 (3)	2.4 ± 0.3 (7)	0.05 ± 0.006 (3)	16.8 ± 1.4 (7)
D333E	3.4 ± 0.5 (6)	0.06 ± 0.005 (3)	2.3 ± 0.3 (6)	0.06 ± 0.003 (3)	15.8 ± 2.5 (6)
E332D/D333E	4.0 ± 0.4 (7)	0.06 ± 0.002 (3)	2.1 ± 0.3 (7)	0.09 ± 0.01 (3)	6.7 ± 0.9 (7)
WM:					
wt	5.2 ± 1.1 (6)	0.26 ± 0.02 (2)	2.9 ± 0.3 (6)	0.10 ± 0.04 (3)	64.8 ± 12.7 (6)
E332A	1.4 ± 0.2 (7)	0.13 ± 0.01 (2)	3.1 ± 0.3 (7)	—[b]	6.1 ± 0.6 (7)
E332D	2.5 ± 0.4 (7)	0.20 ± 0.06 (2)	3.1 ± 0.6 (7)	—[b]	7.2 ± 0.9 (7)
D333A	1.1 ± 0.2 (7)	0.12 ± 0.03 (2)	3.4 ± 0.3 (7)	—[b]	11.0 ± 1.4 (7)
D333E	2.2 ± 0.4 (6)	0.17 ± 0.03 (2)	3.6 ± 0.4 (6)	—[b]	9.2 ± 1.0 (6)
E332D/D333E	2.7 ± 0.7 (7)	0.18 ± 0.03 (2)	3.2 ± 0.2 (7)	—[b]	3.7 ± 0.3 (7)

[a] COS-7 cells expressing LHR (wt, single, and double mutants) were characterized by competitive binding and cAMP responses in BB and WM. B_o = specific binding with 50–400 p*M* ^{125}I-hCG; bcAMP = basal cAMP; mcAMP = maximal cAMP mediated by 100 ng/mL of hCG. Results are given as mean ± SEM for $n = 3–7$ and as average ± range for $n = 2$; n values are given in parentheses.

[b] The dose-response curves are too flat to permit a meaningful determination of the EC_{50} values.

Table 2
Binding and Signaling Characteristics of FSHR in Two Buffers[a]

FSHR	B_o (fmol)	IC_{50} (n*M*)	bcAMP (pmol/mL)	EC_{50} (n*M*)	mcAMP (pmol/mL)
BB:					
wt	3.7 ± 0.2 (5)	0.32 ± 0.03 (3)	3.2 ± 0.4 (5)	0.35 ± 0.04 (3)	27.7 ± 3.8 (5)
E339A	1.2 ± 0.02 (4)	0.48 ± 0.06 (3)	2.0 ± 0.3 (4)	0.85 ± 0.20 (3)	15.2 ± 2.9 (4)
E339D	1.4 ± 0.04 (4)	0.51 ± 0.06 (3)	2.2 ± 0.3 (4)	1.13 ± 0.04 (3)	24.0 ± 4.7 (4)
D340A	2.4 ± 0.4 (4)	0.19 ± 0.04 (3)	2.4 ± 0.3 (4)	0.19 ± 0.06 (3)	21.8 ± 8.7 (4)
D340E	2.8 ± 0.4 (4)	0.30 ± 0.05 (3)	2.3 ± 0.3 (4)	0.18 ± 0.04 (3)	24.9 ± 4.0 (4)
E339D/D340E	1.1 ± 0.2 (4)	0.56 ± 0.35 (2)	2.6 ± 0.4 (4)	0.84 ± 0.29 (3)	11.9 ± 3.2 (4)
WM:					
wt	2.7 ± 0.2 (5)	1.60 ± 0.62 (3)	2.3 ± 0.2 (5)	0.50 ± 0.17 (3)	41.2 ± 6.4 (5)
E339A	0.5 ± 0.04 (4)	2.00 ± 0.70 (3)	3.2 ± 0.3 (4)	—[b]	6.8 ± 0.8 (4)
E339D	0.6 ± 0.01 (4)	0.88 ± 0.38 (2)	3.1 ± 0.3 (4)	2.20 ± 0.10 (2)	11.9 ± 2.3 (4)
D340A	1.4 ± 0.3 (4)	0.87 ± 0.30 (3)	3.0 ± 0.4 (4)	0.63 ± 0.18 (3)	27.6 ± 5.4 (4)
D340E	1.7 ± 0.3 (4)	1.53 ± 0.70 (3)	2.7 ± 0.3 (4)	0.77 ± 0.09 (3)	34.3 ± 4.3 (4)
E339D/D340E	0.7 ± 0.2 (3)	6.00 ± 4.80 (2)	3.4 ± 0.3 (3)	—[b]	7.5 ± 0.6 (3)

[a] Same as footnote to Table 1 except that 50 p*M* ^{125}I-hFSH was used for competitive binding, and maximal cAMP production was determined following addition of 100 ng/mL of hFSH.

[b] The dose-response curves were too flat to permit a meaningful determination of the EC_{50} values.

(i.e., E339A, E339D, and E339D/D340E) and others elevated (i.e., D340A and D340E). The reduced ligand-mediated signaling of the double mutant in BB and WM can be attributable to the reduced receptor levels.

TSHR

The results for TSHR are given in Figs. 2C and 3C and Table 3. Unlike the varying expression levels found for LHR and FSHR (WT and mutants), the expression levels of WT TSHR and TSHR mutants were comparable when assayed in BB. In the presence of WM, the binding was too low to permit accurate assessments of receptor densities and IC_{50}s under the conditions used. In BB, the IC_{50}s and EC_{50}s were similar for WT and mutant TSHRs. EC_{50} values for the mutant forms of TSHR were somewhat greater when assayed in WM than in BB. Basal cAMP production tended

Table 3
Binding and Signaling Characteristics of TSHR in Two Buffers[a]

TSHR	B_o (fmol)	IC_{50} (n*M*)	bcAMP (pmol/mL)	EC_{50} (n*M*)	mcAMP (pmol/mL)
BB:					
wt	1.0 ± 0.2 (4)	0.28 ± 0.07 (4)	10.3 ± 3.1 (4)	0.26 ± 0.02 (3)	36.1 ± 9.5 (4)
E388A	0.6 ± 0.2 (4)	0.25 ± 0.14 (3)	6.6 ± 1.0 (4)	0.29 ± 0.06 (3)	17.6 ± 3.5 (4)
E388D	0.6 ± 0.2 (4)	0.30 ± 0.07 (3)	5.5 ± 1.5 (4)	0.37 ± 0.14 (3)	17.9 ± 6.2 (4)
D389A	1.1 ± 0.3 (4)	0.20 ± 0.07 (4)	8.5 ± 2.0 (4)	0.14 ± 0.04 (3)	26.7 ± 8.5 (4)
D389E	0.9 ± 0.2 (4)	0.29 ± 0.07 (4)	6.2 ± 1.2 (4)	0.19 ± 0.06 (3)	16.1 ± 2.9 (4)
E388D/D389E	0.7 ± 0.2 (4)	0.28 ± 0.05 (3)	5.7 ± 1.1 (4)	0.33 ± 0.13 (3)	14.6 ± 3.1 (4)
WM:					
wt	—[b]	—[b]	4.7 ± 1.3 (4)	0.31 ± 0.07 (2)	76.0 ± 8.6 (4)
E388A	—[b]	—[b]	3.8 ± 1.3 (4)	0.92 ± 0.03 (2)	14.0 ± 3.0 (4)
E388D	—[b]	—[b]	3.8 ± 1.2 (4)	0.87 ± 0.13 (2)	23.8 ± 6.9 (4)
D389A	—[b]	—[b]	4.2 ± 1.2 (4)	0.32 ± 0.10 (2)	36.3 ± 8.3 (4)
D389E	—[b]	—[b]	4.4 ± 1.6 (4)	0.70 ± 0.10 (2)	23.8 ± 4.4 (4)
E388D/D389D	—[b]	—[b]	3.9 ± 1.1 (4)	0.73 ± 0.14 (2)	10.4 ± 2.7 (4)

[a] Same as footnote to Table 1 except that 50 p*M* ^{125}I-bTSH was used for competitive binding, and maximal cAMP production was determined after addition of 100 ng/mL of bTSH.
[b] The low degree of specific binding with 50 p*M* ^{125}I-bTSH prohibited accurate determination of the binding parameters.

to be slightly lower in the presence of the higher ionic strength buffer compared with BB. As with WT LHR and WT FSHR, the maximal TSH-mediated production of cAMP was greater when assayed in WM than in BB; for the single and double mutants, the values were about the same in each buffer. There was no compelling evidence that these TSHR mutants, single and double, represent signaling mutants in BB. In WM, however, the single mutants and the double reversal mutant showed decreased ligand-mediated cAMP production compared to the WT receptor. If we assume similar expression levels, as shown to be the case in BB, then all the mutants behave as signaling mutants, similar to the LHR mutants.

cAMP Production in Transfected Cells Following a Forskolin-Mediated Increase in Adenylyl Cyclase Activity

It has been reported that Na^+ modulates adenylyl cyclase activity *(13,14)*; thus, it was desirable to ascertain whether such an effect could be contributory to our results. Forskolin (100 μ*M*) was added to cells expressing LHR in BB and in WM, and cAMP was determined after a 30-min incubation (37°C) in the presence of 0.8 m*M* isobutylmethylxanthine (IBMX). The results shown in Fig. 4 confirm the higher adenylyl cyclase activity in Na^+-containing buffers. One would therefore expect greater cAMP production in cells maintained in WM compared with BB. Since the mutant LHRs exhibit lower ligand-mediated signaling, this offers additional support for them, particularly the double receptor mutant, to be true signaling mutants.

Discussion

The amino acid sequence FNPCEDIMGY, located in the ectodomain in close proximity to transmembrane helix 1, is identical in the three glycoprotein hormone receptors —LHR, FSHR, and TSHR—and has been suggested to function as a hinge between the LHR ectodomain and endodomain concomitant with ligand binding *(1,2)*. The present study has shown that Glu and Asp in this region, previously reported to be important either directly or indirectly in ligand-mediated LHR signaling but not binding *(1–3)*, seem to be of less functional significance in FSHR and TSHR. Since Na^+ affects the functional characteristics of many G-protein-coupled receptors *(8,15–17)* and the binding of bovine TSH to TSHR is low in the presence of Na^+ *(12)*, the enigmatic role of Na^+ was investigated in these three receptors with a particular emphasis on the possible role of Glu and Asp in mediating some of the reported Na^+ effects. Our results indicate functional differences in this invariant region of the three receptors when assayed in the two buffers of different Na^+ concentration. For example, as judged by the apparent IC_{50} values, there was a reduction in the apparent affinity of all ligands for their cognate receptors in WM compared with BB, perhaps via a kinetic phenomenon; the effect was particularly profound for TSH binding to TSHR. Ligand-mediated signaling of LHR mutants was diminished in WM relative to BB, as it was, to a limited extent, for some of the FSHR mutants.

Since one nonreceptor effect of Na^+ is to enhance adenylyl cyclase activity, one would expect higher levels of

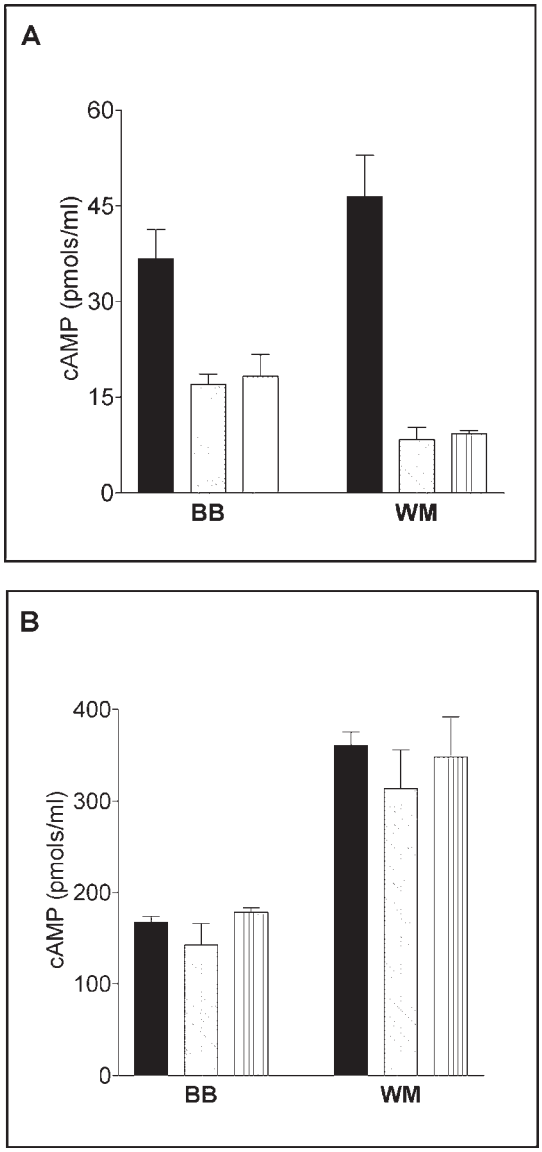

Fig. 4. cAMP production in cells expressing WTLHR (■) and the two mutants, D333A (▧) and D333E (▥), in BB and WM in presence of (**A**) 100 ng/mL of hCG and (**B**) 100 μ*M* forskolin following a 30-min incubation at 37ºC in presence of 0.8 m*M* IBMX. The values in WM are significantly different from those in BB.

cAMP when cells are assayed in the presence of WM compared with BB. However, such a response was not observed in measurements of basal cAMP. Indeed, the higher basal cAMP level associated with TSHR in BB compared with WM is consistent with earlier suggestions that higher ionic strength buffers tend to maintain the receptor in the inactive state *(18,19)*. The three WT receptors exhibited higher levels of ligand-mediated maximal cAMP in WM compared with BB.

One important parameter that is independent of the level of expression is the IC_{50}/EC_{50} ratio, which is a measure of the coupling efficiency between ligand binding and receptor activation. For WT LHR and WT FSHR, this ratio was increased about two- and three- to fourfold, respectively, in WM compared with BB; for TSHR, a meaningful IC_{50} was not readily measurable in WM. Thus, ionic strength (Na^+) exhibits different effects on these three homologous G-protein-coupled receptors, and a functional effect of ionic type or concentration appears to involve Glu-332 and Asp-333 in LHR.

Our results are in partial agreement with another report exploring Na^+ effects on human LHR and TSHR expressed in COS-7 cells in which it was found that, for LHR, the presence of Na^+ reduced basal cAMP levels and led to elevated hCG-stimulated values *(6)*. With rat LHR, also expressed in COS-7 cells, we failed to find an effect of Na^+ on basal cAMP levels but did note a substantial increase in hCG-mediated levels, attributable at least in part to Na^+ enhancement of adenylyl cyclase activity. For TSHR, Cetani et al. *(6)* reported that the presence of Na^+ resulted in lower basal cAMP and higher TSH-stimulated levels; our results are in total agreement with these observations. Other studies have addressed the effect of Na^+ on ovine LH and hCG binding to LHR *(8,9)*. In one study, using MA-10 cells, it was found that Na^+ decreased the binding affinity of ovine LH, but not hCG, to LHR in intact cells and membranes, but not in detergent-solubilized receptors *(8)*. In another study, the LHR mutant D383N (transmembrane helix 2) was investigated using intact HEK 293 cells, and it was observed that the affinity of ovine LH for the LHR mutant was the same in buffers with and without Na^+, and greater than that of hCG *(9)*. By contrast, an effect of Na^+ on the kinetics of hCG binding to LHR was reported earlier *(7)*.

These results with WT LHR, FSHR, and TSHR, as well as selected single and double mutants in this invariant portion of the hinge region between the ectodomain and endodomain, show differential responses of the receptors in low-Na^+- and high-Na^+-containing buffers. Moreover, the Glu and Asp residues located in the invariant sequence exhibit distinct functional roles in the three receptors. Only with LHR and TSHR in the presence of Na^+ is there a convincing argument for an important role of these carboxyl-containing side chains in ligand-mediated receptor activation.

We and others have suggested models for TSHR that invoke an "inactive" or "closed" conformation of the ectodomain, associated with a low level of basal cAMP production, and an "active" or "open" ectodomain conformation, stabilized by TSH binding, associated with a higher level of cAMP production *(20,21)*. The equilibrium between the two forms of TSHR is postulated to be fairly rapid, thus resulting in a higher level of basal cAMP production than that by LHR and FSH, and is presumably influenced by buffer conditions and naturally occurring or engineered mutations. By contrast, LHR and FSHR appear to be stabilized more in the inactive or closed form.

In summary, the identical acidic amino acid residue pair Glu-Asp, located in the conserved region of the ectodomain

proximal to transmembrane helix 1 of the three rat glycoprotein hormone receptors, does not translate into conservation of function.

Materials and Methods

Mutagenesis of LHR, FSHR, and TSHR

Mutagenesis of the rat LHR cDNA, cloned in the expression vector pSVL; the rat FSHR cDNA, cloned in pcDNA3; and the rat TSHR cDNA, cloned in pSG5, was performed by the Quick Change site-directed mutagenesis kit as recommended by Stratagene. Mutant clones were identified by sequencing using a Sequenase Version 2.0 DNA Sequencing Kit (Amersham Pharmacia Biotech, Piscataway, NJ). Mutant cDNAs were amplified, and the Qiagen plasmid maxi kit was used to obtain purified DNA.

Cell Culture and Transfection

COS-7 cells (African green monkey SV40-transformed kidney cells), obtained from American Type Culture Collection (Rockville, MD), were grown in a monolayer culture in Dulbecco's Modified Eagle's Medium (Cellgro, Herndon, VA); supplemented with 10% (v/v) fetal bovine serum (Atlanta Biologicals, Norcross, GA), 50 U/mL of penicillin, 50 µg/mL of streptomycin, 50 µg/mL of gentamycin, and 0.125 µg/mL of amphotericin B; and maintained at 37°C in humidified air containing 5% CO_2. The cells were transiently transfected with 10 µg of the WT or mutant cDNA of each receptor using lipofectamine as recommended by the manufacturer.

Hormone Binding to Transfected Cells

About 16–18 h after transfection, the COS-7 cells were replated (5×10^5 cells/well) into six-well tissue culture plates and assayed for cognate [^{125}I]hormone binding 24 h later: 50–400 p*M* hCG and 50–100 p*M* hFSH (Perkin Elmer, Boston, MA), and 50–100 p*M* bTSH (Covance, Vienna, VA). The competitive binding experiments were performed with ^{125}I-cognate hormone for 6 h at 37°C in the presence of WM, which contains 134 m*M* Na^+, and with BB (278 m*M* sucrose, 5 m*M* HEPES, 1 m*M* $CaCl_2$, 5 m*M* KCl, 1.23 m*M* KH_2PO_4, 1.2 m*M* Mg_2SO_4, 1 m*M* $NaHCO_3$, and 0.1% glucose), which contains 1 m*M* Na^+. Increasing concentrations of unlabeled cognate hormone (hCG, hFSH, or bTSH) were added to each well for these assays. Total binding was corrected for nonspecific binding by addition of a 1000-fold excess of unlabeled cognate ligand, and all results are reported as specific binding.

cAMP Assay in Transfected Cells

About 16–18 h after transfection, the transfected cells were replated (1×10^5 cells/well) into 12-well tissue culture plates. After 24 h, the cells were incubated with increasing or maximal (100 ng/mL) concentrations of cognate hormone (hCG, hFSH, or bTSH) for 30 min at 37ºC in the presence of 0.8 m*M* IBMX (Sigma, St. Louis, MO). Incubation medium was removed and the cells were lysed in 100% ethanol at –20ºC overnight. The extract was collected, dried under vacuum, and resuspended in the cAMP assay buffer for the [^{125}I]cAMP assay kit (Perkin Elmer). cAMP concentrations were determined by radioimmunoassay as recommended by the manufacturer. In one experiment, following the same protocol as just described, forskolin (100 µ*M*) was used to stimulate adenylyl cyclase activity.

Data Analysis

Both binding and signaling (cAMP) data were analyzed by using Prism software (Graph Pad Software, San Diego, CA) using nonlinear regression analysis. All the results represent an average of two to seven independent experiments, each performed in duplicate.

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