Highly Conserved Serine in the Third Transmembrane Helix of the Luteinizing Hormone/Human Chorionic Gonadotropin Receptor Regulates Receptor Activation†

Utpal M. Munshi,‡ Irina D. Pogozheva,§ and K. M. J. Menon*,‡

Departments of Biological Chemistry and Obstetrics/Gynecology, University of Michigan Medical School, Ann Arbor, Michigan 48109-0617, and Department of Medicinal Chemistry, University of Michigan College of Pharmacy, Ann Arbor, Michigan 48109-1065

Received August 19, 2002; Revised Manuscript Received February 7, 2003

ABSTRACT: The elucidation of the role of highly conserved polar amino acids in the transmembrane helices of G-protein-coupled receptors (GPCRs) is important in understanding the mechanism of receptor activation. To this end, the significance of a highly conserved serine residue in the third transmembrane α-helix (TM3) of the luteinizing hormone/human chorionic gonadotropin receptor (LH/hCGR) in regulating receptor activation was examined. Results showed that mutation of serine 431 to alanine (S431A) decreased the ability of the receptor to mediate cAMP production in response to hCG, suggesting that S431 stabilizes the active state of the receptor. Homology with other GPCRs suggests that S431 may participate in the coordination of a Na^+ ion. Since Na^+ has been found to stabilize the active state of the receptor in the presence of hCG, the possibility that S431 promotes receptor activation by mediating the effects of Na^+ was explored. Results showed that the regulation of hormone-induced receptor activation by S431 was independent of Na^+. A rhodopsin-based homology model of the TM region of the LH/hCGR was developed to identify other amino acids that might mediate the effects of Na^+ on receptor function. Results indicate that substitution of an Asp at position 556 with Tyr alters the ability of Na^+ to regulate receptor activation. The homology model is used to explain this result as well as to identify a mechanism through which S431 may regulate receptor signaling. Taken together, these studies provide novel insights into the mechanism of LH/hCG receptor activation.

The luteinizing hormone/human chorionic gonadotropin receptor (LH/hCGR)[1] belongs to the glycoprotein hormone receptor subfamily of rhodopsin-like GPCRs (*1*). The receptor is primarily expressed in reproductive tissues and regulates physiological functions such as spermatogenesis, ovulation, and pregnancy (*1*−*3*). The LH/hCGR is 674 amino acids in length and contains an exodomain and an endodomain approximately equal in size (*2*, *3*). The exodomain is glycosylated and consists of the extracellularly located N-terminal portion of the receptor, while the endodomain is comprised of seven transmembrane α-helices, intracellular and extracellular loops, and a C-terminal cytoplasmic tail (*2*−*5*). The leucine-rich repeat containing exodomain binds LH or hCG with high affinity, whereas the endodomain is believed to transduce the activation signal to G-protein (*2*, *6*−*8*). Receptor activation results in cAMP production and, at higher hormone concentrations, leads to the production of inositol phosphates (*9*−*12*). While the physiological importance of LH/hCGR-mediated cAMP production has been well-established, the significance of receptor-mediated inositol phosphates accumulation is not well understood (*13*).

GPCR activation is believed to involve a movement and rotation of the transmembrane α-helices (TM), especially TM3 and 6, that may open a cytoplasmic cleft in the receptor and subsequently facilitate interaction with G-protein (*14*−*21*). The most striking examples consistent with this notion are the many naturally occurring point mutations in the TM that lead to pathophysiological conditions. For example, mutations of amino acid residues in TM6 of the human LH/hCGR cause constitutive activation, resulting in familial male precocious puberty, whereas LH/hCGR TM mutants unable to undergo activation cause conditions such as Leydig cell hypoplasia (*31*, *32*). Conditions such as retinitis pigmentosa and congenital night blindness have been shown to arise from TM mutations that cause constitutive activation of rhodopsin (*24*, *25*). Similar examples from other GPCRs include the TSHR, where mutations in the TM causing constitutive receptor activation result in thyroid adenomas (*26*).

In addition to point mutations in the TM, Na^+ has also been shown to play a role in LH/hCGR activation. Specifically, replacement of extracellular Na^+ with substitutes has been shown to result in constitutive activation of the LH/

† Supported by National Institutes of Health Grant HD-06656. I.D.P. is supported by National Institutes of Health Grant R21 GM61299. U.M.M. is a predoctoral fellow supported by National Institutes of Health Training Grant PR5-T32 HD-07048.

* To whom correspondence should be addressed: University of Michigan Medical School, 6428 Medical Sciences Building I, 1301 Catherine St., Ann Arbor, MI 48109-0617. Telephone: (734) 764-8142. Fax: (734) 936-8617. E-mail: kmjmenon@umich.edu.

‡ University of Michigan Medical School.

§ University of Michigan College of Pharmacy.

[1] Abbreviations: LH/hCGR, luteinizing hormone/human chorionic gonadotropin receptor; GPCR, G-protein-coupled receptor; LH, luteinizing hormone; hCG, human chorionic gonadotropin; TM, transmembrane α-helix; TSHR, thyroid stimulating hormone receptor; NMDG, *N*-methyl-D-glucamine; TMA-Cl, tetramethylammonium chloride; TMA-OH, tetramethylammonium hydroxide; DMEM, Dulbecco's modified Eagle's medium; TMA^+, tetramethylammonium ion; $NMDG^+$, *N*-methyl-D-glucamine ion; IBMX, isobutylmethylxanthine; RIA, radioimmunoassay; SEM, standard error of the mean.

10.1021/bi026672e CCC: $25.00
Published on Web 03/12/2003

hCGR and reduced hCG-stimulated cAMP production (*27*, *28*). A similar phenomenon has been observed for the TSHR (*28*). Na^+ has also been shown to stabilize the inactive state of a several other GPCRs (*29*, *30*). Though the site(s) at which Na^+ regulates LH/hCGR activation is unknown, D383 of TM2 of the rat LH/hCGR has been shown to mediate Na^+ regulation of LH binding affinity (*27*). Thus, it has been suggested that Na^+ may be coordinated to D383 (*27*). In fact, previous studies have proposed that this highly conserved aspartic acid residue may mediate sodium's effects on ligand binding and/or receptor activation in other GPCRs (*31*−*33*). Since Na^+ is usually coordinated by six oxygen atoms (*34*), water molecules and/or amino acids in the vicinity of D383 of the LH/hCGR may also participate in the coordination of Na^+. Supporting this notion is a recent study on the dopamine D2 receptor (*33*) which suggested that in addition to an aspartic acid residue homologous to D383 in TM2, a conserved serine in TM3 as well as a conserved asparagine in TM7 could be involved in the formation of a Na^+ binding pocket.

The recently obtained X-ray structure of bovine rhodopsin at 2.8 Å resolution provides a template for homology modeling of other members of the rhodopsin-like family of GPCRs (*35*) and gives insights into the structural basis of receptor activation (*35*). The rhodopsin structure indicates the presence of a core four-α-helix bundle consisting of transmembrane helices 2, 3, 6, and 7 (*35*). Many naturally occurring as well as experimentally induced mutations that regulate LH/hCGR activation are located in the putative four-α-helix bundle, indicating the possible significance of these four helices in regulating the activation process. For example, in addition to the previously mentioned mutations in TM6 of the LH/hCGR that result in pathophysiological conditions (*23*), mutation of asparagine residues in TM7 has also been shown to regulate LH/hCGR activation (*36*). Furthermore, the highly conserved aspartic acid in TM2 (D383) has been shown to regulate receptor affinity for hCG as well as the EC_{50} for hCG-induced cAMP production (*27*, *37*). While the importance of TM3 in receptor signaling has been studied extensively for other GPCRs (*17*, *20*, *38*), only three residues in TM3 of the LH/hCGR in addition to the glutamate and arginine residues of the highly conserved E/DRW/Y motif have been examined with respect to their effects on receptor activation (*36*, *37*, *39*−*42*). Of these residues, only L435 has been found to be important, as substitution of this leucine with positively charged amino acids results in constitutive activation of the receptor (*39*). Results from previous modeling studies suggest that a highly conserved serine in TM3 of the rat LH/hCGR (S431) may play a role in receptor activation (*21*, *43*). As the functional importance of S431 has not been investigated in the LH/hCGR, this study examined the role of this residue in LH/hCGR-mediated signaling.

EXPERIMENTAL PROCEDURES

Materials. Human chorionic gonadotropin (CR-127) was a gift from the Center for Population Research (National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD). $Na^{125}I$ was purchased from ICN. Human embryonic kidney cells (293T cells) expressing the large T antigen were a gift from Dr. G. P. Nolan (Stanford University, Stanford, CA). hCG for non-specific binding and NMDG were purchased from Sigma. Cell culture media were purchased from Gibco BRL. The [^{125}I]cAMP RIA kit was purchased from Amersham Pharmacia. TMA-Cl and TMA-OH were purchased in powder form from ICN. The pCMV4 plasmid was generously provided by Dr. D. Russell (University of Texas Southwestern Medical Center, Dallas, TX). Mutant receptor cDNA was prepared using Promega's Gene Editor in vitro site-directed mutagenesis system. All statistical analyses were performed using the computer program PRISM.

Cell Culture and Transfection. Experiments were performed using human embryonic kidney cells expressing the large T antigen (293T). 293T cells were grown in DMEM containing 4500 mg/L glucose, 50 mg/mL gentamycin, 9 units/mL nystatin, 10 mM HEPES, and 10% fetal bovine serum at 37 °C under conditions described previously (*10*). Exponentially growing 293T cells ($2-3 \times 10^6$ cells) were plated in 100 mm dishes approximately 5 h prior to transient transfection. Transfection was performed using the calcium phosphate coprecipitation method. Cells were harvested 60 h following transfection.

[^{125}I]hCG Binding Assay. [^{125}I]hCG binding assays were performed as described previously (*10*). The K_d of hCG binding for the wild-type (WT) and S431A mutant receptor was determined by incubating 293T cells with 0.08−4 nM [^{125}I]hCG as described previously (*44*). To determine the level of cell surface receptor expression (B_{max}), cells were incubated with a 120 ng/mL saturating concentration of [^{125}I]-hCG (specific activity = 40−60 cpm/pg) at 4 °C for 16 h. The level of nonspecific binding was determined by including a 1000-fold excess of unlabeled hCG in the binding reaction. Twenty hours later, cells were washed twice with Waymouth's medium at 4 °C to remove unbound hormone. The [^{125}I]hCG associated with the pellet was counted in a Gamma Trac 1290 γ-counter (Tm Analytic).

Measurement of cAMP Production. Transiently transfected 293T cells were harvested with either PBS-EDTA, buffer A (0.8 mM $CaCl_2 \cdot 2H_2O$, 2 mM KCl, 1.2 mM $MgCl_2 \cdot 6H_2O$, 0.8 mM $MgSO_4$, 100−130 mM NaCl, 25 mM Hepes, 28 mM D-glucose, and 0.1% BSA, adjusted to pH 7.4 with NaOH), or buffer B [same as buffer A, except that the NaCl was substituted with the appropriate amounts of tetramethylammonium chloride (TMA-Cl) or *N*-methyl-D-glucamine (NMDG) such that after adjusting the pH to 7.4 with TMA-OH or HCl, respectively, the concentration of Na^+ in buffer A would equal the concentration of TMA^+ or $NMDG^+$ in buffer B]. While the concentration of Na^+ varied from experiment to experiment, the concentration of Na^+ was equal to that for TMA^+ or $NMDG^+$ in a given experiment. The cells were then resuspended in assay buffer (Waymouth's MB 752/1, buffer A, or buffer B). cAMP measurements depicted in Figure 1 and Table 1 were performed in Waymouth's MB 752/1 medium (Na^+ concentration in Waymouth's is 100 mM), and Na^+ sensitivity experiments presented in Figures 2, 3, 5, and 6 and Tables 2−4 were performed in buffer A or buffer B, as appropriate. Cells were subsequently preincubated with 500 μM IBMX at 37 °C for 15 min, followed by stimulation with the indicated concentration of hCG for 30 min at 37 °C. The stimulation was terminated by the addition of 1.25 mL of Waymouth's MB752/1, buffer A, or buffer B at 4 °C and vortexing, followed by centrifugation at 200*g* for 5 min at 4 °C. The

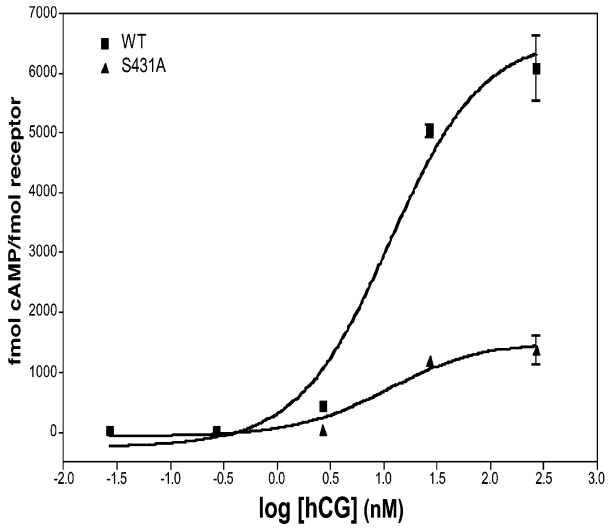

FIGURE 1: cAMP dose−response curve for 293T cells expressing the WT or S431A LH/hCGR. 293T cells were transiently transfected with cDNA expressing the WT or mutant receptor. Quantities of cDNA resulting in comparable cell surface receptor expression were transfected (4.0 μg of S431A cDNA or 1.75 μg of WT cDNA). The empty pCMV4 vector was also transfected to equalize the mass of DNA added to each plate. Sixty hours after transfection, cells were harvested and cAMP assays performed as described in Experimental Procedures. Results shown are from one experiment (average of duplicate measurements ± SEM). If an error bar does not appear on a given data point, the SEM was too small to graph.

Table 1: Cell Surface hCG Binding and hCG-Induced cAMP Production in 293T Cells Expressing the WT or S431A LH/hCGR

LH/hCGR construct	B_{max}[a] (fmol of receptor/ μg of total protein)	K_d (nM)	maximal cAMP (%)
WT	0.33 ± 0.10 (n = 5)	0.95 ± 0.31 (n = 2)	100 (n = 5)
S431A	0.50 ± 0.15 (n = 5)	1.17 ± 0.19 (n = 2)	46 ± 10[b] (n = 5)

[a] Determined at a saturating [^{125}I]hCG concentration of 3.2 nM. [b] Significantly different from WT as determined by a one-sample t test ($P < 0.05$).

cells were then washed in PBS/EDTA and subsequently resuspended in 400 μL of 100% ethanol at −20 °C. Cells were vortexed and subsequently incubated for 21 h at −20 °C to extract cAMP. At the end of this incubation, the ethanol was evaporated and the total amount of cAMP in the remaining cellular residue was measured using the [^{125}I]-cAMP RIA kit from Amersham Pharmacia. cAMP production was normalized for receptor expression by expressing the data in units of femtomoles of cAMP per femtomole of receptor, where the number of femtomoles of cAMP per femtomole of receptor equals the number of femtomoles of cAMP produced per mass of cell surface receptor. Receptor mass was calculated from B_{max} values measured for WT and mutant receptors. B_{max} values were determined as described above. The figures show data from the number of experiments indicated in the corresponding figure legend. The tables present a summary of data from all experiments.

Molecular Modeling of the Transmembrane Region of the Rat LH/hCGR. The atomic model of the seven-α-helix transmembrane fragment, amino acids 335−621 of the rat LH/hCGR,[2] was built by homology to bovine rhodopsin using the Protein Data Bank coordinates (*45*) of the template protein (1f88), the alignment of sequences of both proteins from the GPCR Database (*46*), and a program we have developed that substituted all side chains of bovine rhodopsin for the corresponding residues of the LH/hCGR, taking the main chain dihedral angles (φ and ψ) and the side chain χ^1 angles from the template as was done for modeling of opioid receptors (*47*). During initial modeling, the α-aneurisms were removed from TM5 of the rhodopsin template and several residues that are missing in the rat LH/hCGR sequence, corresponding to rhodopsin residues 182−184, 196−198, and 234−239, were deleted from the structure. A QUANTA visualization module (Molecular Simulations Inc.) was used for the manual adjustment of the side chain rotamers to remove major hindrances and to form H-bonds between polar side chains.

The initial model was refined as described previously (*47*) by distance geometry with the program DIANA (*48*) using the lists of distance and angle constraints generated from the template as well as the intrahelical H-bonds (upper limit for the $NH_i\cdots O_{i-4}$ distance being 1.9 Å) between polar side chains of the receptor that were obtained from the crude homology model. This allowed the removal of steric hindrances between tightly packed side chains leaving major intra- and interhelical distances and angle constraints similar to the rhodopsin template with a 0.5−1 Å deviation for Cβ−Cβ distances (at a proximity of <8 Å) and a 20° deviation for main chain and side chain angles. During the refinement procedure, residues 407−413 (NHAIDWQ) as well as L443, which do not have equivalents in the rhodopsin structure, were inserted between residues F105 and G106 in extracellular loop 1 and between residues S144 and N145 in intracellular loop 2 of rhodopsin. The residues in the region between TM5 and -6 corresponding to the flexible region of residues 230−243 in the rhodopsin crystal structure (*35*) were constrained at the α-helical conformation for residues 230−232, 242, and 243, in accordance with cysteine modification data (*49*), spin-labeling studies (*50*), and disulfide cross-linking results (*51*). The backbone angles for other residues in the area of insertions were unconstrained. The naturally present disulfide bond between C417 and C492 was explicitly introduced into the DIANA calculation together with the list of 665 distance and 1036 angle constraints, taken from the initial model of the rat LH/hCGR. All aspartic acid, glutamic acid, arginine, and lysine residues were considered charged during calculations. The standard target function minimization strategy (*47*) was used to calculate the rmsd between calculated structures and the distances between possible H-bond partners in different models. The calculation was finished at a target function of <40 (for the TM fragment of 288 residues). The five structures with the lowest target function from a total of 50−150 trial structures were analyzed with QUANTA. These five structures converged well with the pairwise rmsd for TM residue C_α atoms (<1.0 Å).

RESULTS

Effect of the Mutation of S431 on Basal and Maximal Hormone-Induced cAMP Production. To examine the importance of serine 431 in regulating receptor activation, this

[2] Swiss-Prot entry P22888, with 22 excluded residues of signal sequence.

residue was mutated to alanine (S431A), and the levels of basal and maximal hormone-induced cAMP production were measured in transiently transfected human embryonic kidney cells (293T cells) expressing this receptor. Appropriate amounts of cDNA encoding WT or mutant receptor were transfected to give approximately equivalent levels of cell surface receptor expression (B_{max}) as examined by the [^{125}I]-hCG binding assay (Table 1). Data from cAMP measurements were normalized for receptor expression to correct for variation in this parameter from experiment to experiment.

The hCG dose−response data from a single experiment shown in Figure 1 indicate that both the WT and S431A mutant receptor responded to increasing concentrations of hCG with an increase in the level of cAMP production. However, the maximal level of hCG-stimulated cAMP production is lower in cells expressing S431A mutant receptor than in cells expressing the WT receptor. Basal cAMP production showed only minor differences between the WT and S431A mutant receptor (data not shown).

Table 1 summarizes the data from all experiments that examined hormone-induced cAMP production mediated by the WT and S431A mutant receptor. The results show a statistically significant reduction in the maximal level of cAMP production mediated by the S431A mutant receptor, suggesting that S431 is important in stabilizing the active state of the receptor under hormone-induced conditions. Since the K_d for hCG binding was similar for the WT and S431A mutant receptor (0.95 ± 0.31 and 1.17 ± 0.19 nM, respectively) (Table 1), this indicates that the decrease in the hCG-induced cAMP response in cells expressing the S431A mutant receptor is not due to altered hCG binding affinity.

Effects of Na^+ on WT- and S431A-Mediated cAMP Production. Ligand binding studies on the dopamine D2 receptor (*33*) suggest that the serine residue homologous to S431 of the LH/hCGR participates in the coordination of a Na^+ ion together with a conserved aspartate of TM2 (corresponding to D383 of the LH/hCGR). Taken together with the fact that replacement of extracellular Na^+ has been shown to promote hormone-induced LH/hCGR activation (*28*), it is possible that S431 may promote receptor activation by mediating the effects of Na^+. To test this possibility, the consequences of replacement of extracellular Na^+ on basal and hormone-induced cAMP production mediated by the WT or S431A mutant receptor were examined in 293T cells incubated in medium containing a physiological concentration of Na^+ (buffer A) or in medium containing an equivalent concentration of the sodium substitutes TMA^+ or $NMDG^+$ (buffer B) (*53*). The concentration of Na^+ in buffer A was similar to the concentration of Na^+ in Waymouth's MB 752/1 medium (100 mM). The average level of cell surface receptor expression (B_{max}) for this set of experiments as measured by [^{125}I]hCG binding assays was comparable for the WT and mutant receptor (data not shown). Data were normalized for receptor expression to correct for any variation in this parameter from experiment to experiment.

The results presented in Figure 2 indicate that the replacement of extracellular Na^+ results in an increase in basal cAMP levels in cells expressing the WT receptor. A summary of all experiments presented in Table 2 indicates that substitution of Na^+ yielded statistically significant

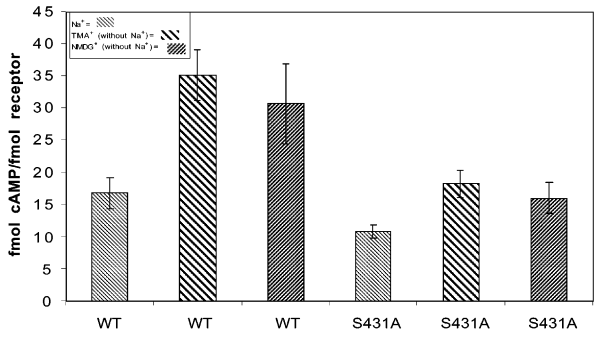

FIGURE 2: Basal S431A signaling in the presence or absence of Na^+. 293T cells were transiently transfected with cDNA expressing the WT or S431A mutant receptor. Quantities of cDNA were transfected to result in comparable cell surface receptor expression (4.0 μg of S431A cDNA or 1.75 μg of WT cDNA). The empty pCMV4 vector was also transfected to equalize the mass of DNA added to each plate. Sixty hours after transfection, cells were harvested, washed, and resuspended in Na^+-, TMA^+-, or $NMDG^+$-containing buffer. After incubation of cells at 37 °C with IBMX for 15 min, cells were incubated for a further 30 min at 37 °C. At the end of the incubation period, cells were washed, and cAMP production was measured as described in Experimental Procedures. The data presented are the average of five independent experiments ± SEM.

Table 2: Summary of Effects of Na^+ Replacement on Basal WT and S431A Signaling[a]

		fold increase in cAMP[b]	
substitute	*n*	WT	S431A
TMA^+	9	2.46 ± 0.58 ($P < 0.05$)	1.54 ± 0.13 ($P < 0.05$)
$NMDG^+$	9	1.85 ± 0.32 ($P < 0.05$)	1.37 ± 0.15 ($P < 0.05$)

[a] Statistical significance from a value of 1 tested using a one-sample *t* test. [b] Fold increase in cAMP is the level of basal cAMP production in the presence of TMA^+ or $NMDG^+$ divided by the level of basal cAMP production in the presence of Na^+.

increases in the level of WT and S431A mutant receptor-mediated cAMP production, although the increases were larger for the WT receptor. This leads to the conclusion that under basal conditions, the S431A mutant receptor has lost sensitivity to Na^+ substitution as compared to the WT receptor. These results also suggest that S431 may play a minor role in regulating the ability of Na^+ to stabilize the inactive state of the receptor. The results presented in Figure 3 and a summary of all experiments presented in Table 3 show a decrease in the maximal level of WT or mutant receptor signaling upon replacement of extracellular Na^+ with TMA^+ or $NMDG^+$. The extent of the decreases in cAMP production upon replacement of Na^+ is similar for the WT and S431A mutant receptor. This suggests that S431 does not regulate hormone-induced receptor activation through a mechanism involving Na^+. It is unlikely that the decrease in the level of hormone-induced signaling for the WT and mutant receptor upon substitution of Na^+ is a result of an effect of Na^+ on the K_d for hCG binding, as Na^+ has been shown to have no effect on this parameter (*53*). Since the effects of extracellular Na^+ replacement with either substitution reagent on basal and hormone-induced cAMP production were comparable, the phenomena that were seen were due to Na^+ depletion rather than the effects of the type of Na^+ substitute that was used.

Effects of Na^+ on Basal D556G and D556Y Signaling. To identify other residues that may be involved in mediating

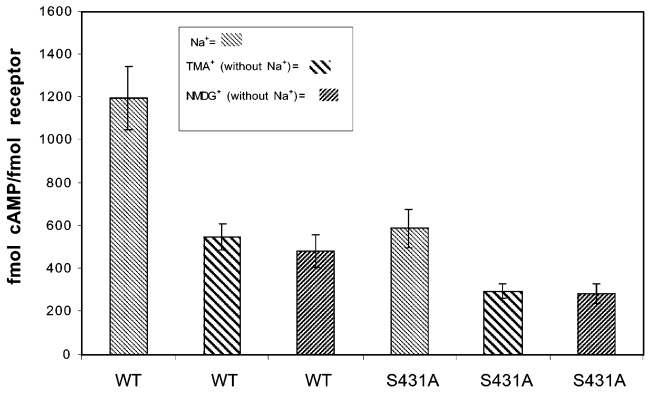

FIGURE 3: Maximal S431A signaling in the presence or absence of Na^+. 293T cells were transiently transfected with cDNA expressing the WT or S431A mutant receptor. Quantities of cDNA were transfected to result in comparable levels of cell surface receptor expression (4.0 μg of S431A cDNA or 1.75 μg of WT cDNA). The empty pCMV4 vector was also transfected to equalize the mass of DNA added to each plate. Sixty hours after transfection, cells were harvested, washed, and resuspended in Na^+-, TMA^+-, or $NMDG^+$-containing buffer. After incubation of cells at 37 °C with IBMX for 15 min, cells were incubated for a further 30 min at 37 °C with 10000 ng/mL hCG. At the end of the incubation period, cells were washed, and cAMP production was measured as described in Experimental Procedures. The data presented are the average of four independent experiments ± SEM.

Table 3: Summary of Effects of Na^+ Replacement on Maximal WT and S431A Signaling[a]

		fold decrease in cAMP[b]	
substitute	*n*	WT	S431A
TMA^+	7	0.48 ± 0.09 ($P < 0.05$)	0.51 ± 0.06 ($P < 0.05$)
$NMDG^+$	6	0.36 ± 0.06 ($P < 0.05$)	0.50 ± 0.05 ($P < 0.05$)

[a] Statistical significance from a value of 1 tested using a one-sample *t* test. [b] Fold decrease in cAMP is the level of maximal cAMP production in the presence of TMA^+ or $NMDG^+$ divided by the level of maximal cAMP production in the presence of Na^+.

the effects of Na^+ on receptor signaling, a rhodopsin-based homology model of the inactive state of the rat LH/hCGR was developed as described in Experimental Procedures (Figure 4). It should be noted that all five residues depicted in this model (D383 from TM2, S431 from TM3, D556 from TM6, and N593 from TM7, and N597 from TM7) are also present in the human LH/hCGR. Furthermore, the rat and human forms of this receptor are approximately 90% identical at the amino acid level in the TM region as well as the overall receptor (*13*). The model indicates that S431, D556, N593, and N597 are close to D383, a potential Na^+ coordination site (*27*). The spatial proximity of D556 to D383 along with its negative charge suggests that D556 may coordinate Na^+. Furthermore, mutations of D556, such as D556G and D556Y, have been shown to result in higher levels of basal cAMP production compared to that of the WT receptor (*52*) [D556G and D556Y are rat homologues of naturally occurring constitutively active D578G and D578Y mutations found in patients with familial male precocious puberty (*23*, *54*)]. Since D556 is close to D383, and because Na^+ might stabilize the inactive state of the receptor (Figure 2 and Table 2) (*7*, *28*), it is possible that the higher level of basal cAMP production of the D556 mutants as compared to that of the WT receptor may be due to an altered ability of Na^+ to regulate receptor activation.

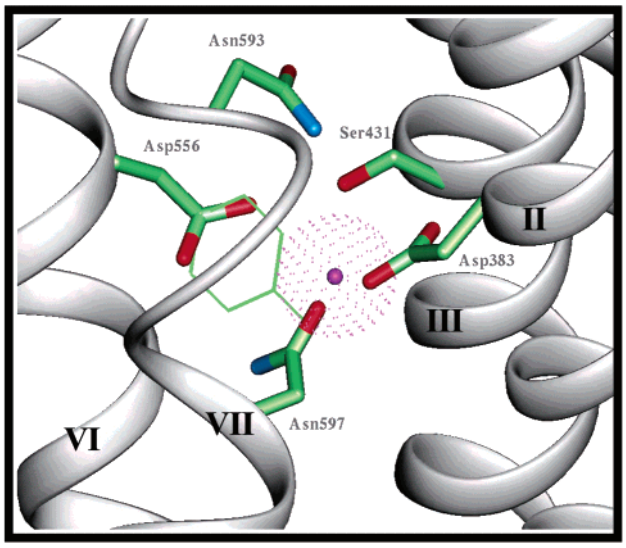

FIGURE 4: Model of S431 and surrounding residues in the inactive conformation of LH/hCGR. The model was developed as described in Experimental Procedures. Serine 431 is depicted along with proximal polar residues. Gray ribbons represent backbone α-carbons of a TM helix. The helix number is shown in the ribbon in black, bold roman numerals. Side chain carbon atoms are colored green. Oxygen atoms are colored red. Nitrogen atoms are colored blue. The solid purple sphere and surrounding purple dots together represent a Na^+ ion. Light green lines at position 556 represent a Tyr side chain.

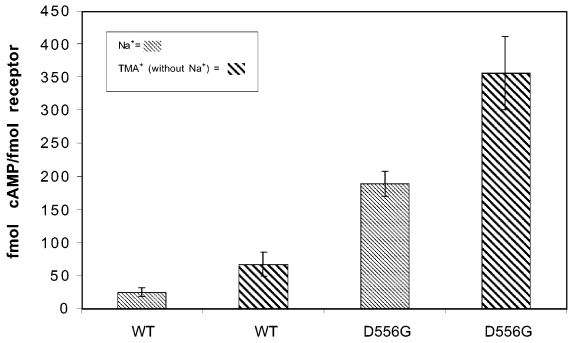

FIGURE 5: Basal D556G signaling in the presence or absence of Na^+. 293T cells were transiently transfected with cDNA expressing the WT, D556G, or D556Y mutant receptor (2.0 μg of WT or 10.0 μg of D556G). The empty pCMV4 vector was also transfected to equalize the mass of DNA added to each plate. Sixty hours after transfection, cells were harvested, washed, and resuspended in Na^+-, TMA^+-, or $NMDG^+$-containing buffer. After incubation of cells at 37 °C with IBMX for 15 min, cells were incubated for a further 30 min at 37 °C. At the end of the incubation period, cells were washed, and cAMP production was measured as described in Experimental Procedures. The data presented are the average of four independent experiments ± SEM.

To test this hypothesis, the effect of replacement of extracellular Na^+ with TMA^+ on basal signaling of D556G and D556Y mutant receptors was examined. The level of D556Y cell surface receptor expression (B_{max}) was comparable to that for the WT receptor, while that for D556G was 60% lower than that for the WT receptor (data not shown). Data were normalized for receptor expression. Consistent with previous observations, the data presented in Figures 5 and 6 indicate that in the presence of Na^+, both D556G and D556Y display higher levels of basal cAMP production than the WT receptor (*52*). As seen in the summary of all experiments presented in Table 4, replacement of extracellular sodium with TMA^+ increases basal cAMP production

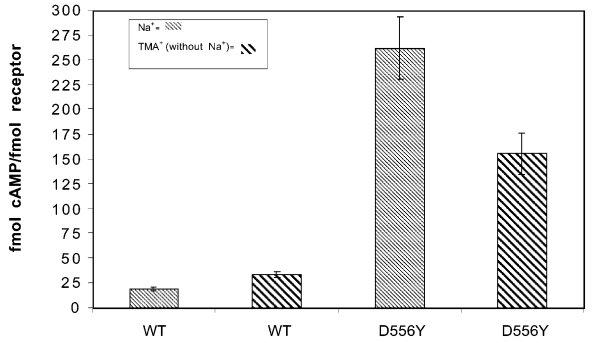

FIGURE 6: Basal D556Y signaling in the presence or absence of Na^+. 293T cells were transiently transfected with cDNA expressing the WT or D556Y mutant receptor (2.0 μg of WT or 3.5 μg of D556Y). The empty pCMV4 vector was also transfected to equalize the mass of DNA added to each plate. Sixty hours after transfection, cells were harvested, washed, and resuspended in Na^+-, TMA^+-, or $NMDG^+$-containing buffer. After incubation of cells at 37 °C with IBMX for 15 min, cells were incubated for a further 30 min at 37 °C. At the end of the incubation period, cells were washed, and cAMP production was measured as described in Experimental Procedures. The data presented are the average of two independent experiments ± SEM.

Table 4: Summary of Effects of Na^+ Replacement on Basal D556G and D556Y Signaling[a]

		fold increase in cAMP[b]	
substitute	*n*	WT	D556G
TMA^+	7	2.78 ± 0.64 ($P < 0.05$)	1.97 ± 0.27 ($P < 0.05$)
		fold increase in cAMP[b]	
substitute	*n*	WT	D556Y
TMA^+	4	1.93 ± 0.26 ($P < 0.05$)	0.72 ± 0.08 ($P < 0.05$)

[a] Statistical significance from a value of 1 tested using a one-sample *t* test. [b] Fold increase in cAMP is the level of basal cAMP production in the presence of TMA^+ divided by the level of basal cAMP production in the presence of Na^+.

mediated by the WT or D556G mutant receptor [2.78 ± 0.64 ($P < 0.05$) or 1.97 ± 0.27 ($P < 0.05$), respectively] (Table 4). Interestingly, replacement of Na^+ significantly decreased the level of D556Y-mediated basal cAMP production [TMA^+/Na^+ is 0.72 ± 0.08 ($P < 0.05$)] (Table 4). Replacement with $NMDG^+$ had effects similar to those of replacement with TMA^+ on D556G and D556Y basal cAMP production (data not shown).

DISCUSSION

This study is the first examination of the LH/hCGR with respect to the functional importance of a highly conserved serine residue in the third transmembrane helix, S431. The data show that while the S431A mutant displays unaltered hCG binding affinity, it has a reduced ability to mediate maximal cAMP production. This indicates that S431 participates in stabilizing the active state of the receptor. While most studies on other GPCRs have found a functional role for this serine in regulating ligand binding affinity (*55*, *56*), studies on only two members of the GPCR family suggest a role for this serine in regulating receptor activation (*38*, *57*).

The simplest explanation for the reduced ability of the S431A mutant to mediate maximal cAMP production is that the hydroxyl group of S431 participates in a hydrogen bonding network that stabilizes the active state of the receptor. This prediction follows from the concept that receptor activation may involve the disruption of a hydrogen bonding network that stabilizes the inactive state while promoting the formation of a new set of hydrogen bonds that stabilizes the active state (*13*, *38*, *57*). The model of the inactive state of the LH/hCGR presented in Figure 4 gives insights into the nature of the interhelical interactions in which S431 may participate.

Figure 4 shows a cluster of polar residues (D383, D556, N593, and N597) surrounding a cavity that contains a Na^+ ion coordinated to D383 (*27*). This cavity can also accommodate water molecules. The distances between the polar groups indicate that the only moiety within hydrogen bonding distance of the hydroxyl group of S431 in the inactive state is the side chain carboxyl group of D556 (the pairwise distance between $O^{\delta 2}$ of D556 and O^{γ} of S431 was 2.4−3.2 Å). The pairwise distances between polar groups of other residues presented in Figure 6 are greater than the standard upper limits for H-bond formation (2.9 Å for N···O or O···O distances). For example, the distance between $O^{\delta 1}$ of D556 and $N^{\delta 2}$ of N597 is 4.3−4.8 Å, between O^{γ} of S431 and $N^{\delta 2}$ of N597 is 4.0−4.8 Å, between O^{γ} of S431 and $N^{\delta 2}$ of N593 is 3.7−5.3 Å, between O^{γ} of S431 and $O^{\delta 2}$ of D383 is 5.0−5.9 Å, and between $O^{\gamma 1}$ of N597 and $O^{\delta 1}$ of D383 is 3.4−4.8 Å. If a hydrogen bond between S431 and D556 stabilizes the inactive state, it would follow that the S431A mutant should display higher basal activity than the WT receptor. As this was not the case, it is unlikely that a hydrogen bonding interaction between these two residues prevents receptor activation. Since S431A is not constitutively active, it is also unlikely that any hydrogen bonding interactions that S431 might form indirectly through water with D383, N593, or N597 stabilize the inactive state of the receptor.

Since GPCR activation could involve movement of TM3 and TM6 (*17*, *58*)**,** it is conceivable that receptor activation results in movement of S431 away from D556 and toward the polar groups of D383, N593, or N597 such that S431 may interact through hydrogen bonding with any of these three residues to stabilize the active state LH/hCGR. This interaction may occur directly by hydrogen bonding between the side chains of the two amino acid residues or indirectly through hydrogen bonding with water molecules in the cavity. Due to the absence of a template structure for the active state of a GPCR, a reliable model cannot be built to verify the hydrogen bonding partner of S431 in the active state LH/hCGR. However, the following analysis of previously published substitution mutants elucidates the possible identity of such an amino acid.

Previous studies indicate that D556 participates in stabilization of the inactive as opposed to the active state of the receptor (*59*). Thus, it is unlikely that an interaction between D556 and S431 promotes receptor activation. Additionally, it has been shown that certain substitution mutations of N593, N597, and D383 resulted in diminished signaling (*27*, *36*, *37*). Since an N597A mutant LH/hCGR has been shown to maintain WT cAMP signaling capability (*36*), it is improbable that a hydrogen bonding interaction between S431 and N597 is important in stabilizing the active state. Results from the reciprocal mutation of residues corresponding to D383 and N597 in several GPCRs indicate the importance of a putative interaction between these two highly conserved

residues in promoting receptor activation (*60*, *61*). Findings of Angelova et al. (*36*) indicate that an N593A mutant LH/hCGR displays severely reduced cAMP production as compared to the WT receptor while displaying unaltered hCG binding affinity. Taken together, these studies suggest that the most likely candidate for the active state hydrogen bonding partner of S431 is N593 in TM7.

The replacement of Na^+ produced no increase in the level of basal cAMP production for the D556Y mutant receptor. This suggests that Na^+ has completely lost its ability to stabilize the inactive state of the D556Y mutant receptor. The model presented in Figure 4 suggests that substitution of a tyrosine at position 556 (D556Y) may reduce the size of the cavity. The altered conformation produced by tyrosine substitution at position 556 may prevent Na^+ from entering the cavity to stabilize the inactive state. Interestingly, it has been shown that GPCRs containing a phenylalanine at the position corresponding to position 556 of the LH/hCGR display sensitivity to Na^+ regulation of basal receptor activation similar to that for the WT LH/hCGR (*30*, *62*, *63*). This suggests that the hydroxyl group of a tyrosine at position 556, rather than the phenyl ring itself, restricts Na^+ access to the cavity. The statistically significant decrease in the level of D556Y basal signaling upon replacement of Na^+ also suggests that blocking Na^+ access to the cavity unmasks stimulatory effects that Na^+ may have at other site(s) in the signaling pathway. This is consistent with the results presented in Figure 3 and Table 3. Interaction of hormone with receptor may change the size of the cavity and consequently prevent Na^+ entry, thereby unmasking the stimulatory effects of Na^+ on signaling.

In conclusion, the results show that a highly conserved serine in the third transmembrane helix stabilizes the active state of the LH/hCGR and suggest a structural foundation for the role of this residue in regulating receptor activation. Furthermore, these data provide a molecular basis for the participation of Na^+ in the LH/hCGR activation process. Taken together, these studies provide a working model for elucidating the mechanism of LH/hCGR activation.

ACKNOWLEDGMENT

We thank Dr. Anil Nair, Dr. P. K. Pradeep, Christine Clouser, and Helle Peegel for helpful discussions as well as a critical reading of the manuscript.

REFERENCES

1. Dufau, M. L. (1998) *Annu. Rev. Physiol. 60*, 461−496.
2. McFarland, K. C., Sprengel, R., Phillips, H. S., Kohler, M., Rosemblit, N., Nikolics, K., Segaloff, D. L., and Seeburg, P. H. (1989) *Science 245*, 494−499.
3. Loosfelt, H., Misrahi, M., Atger, M., Salesse, R., Vu Hai-Luu Thi, M. T., Jolivet, A., Guiochon-Mantel, A., Sar, S., Jallal, B., and Garnier, J. (1989) *Science 245*, 525−528.
4. Zhang, R., Cai, H., Fatima, N., Buczko, E., and Dufau, M. L. (1995) *J. Biol. Chem. 270*, 21722−21728.
5. Vuhai-Luuthi, M. T., Jolivet, A., Jallal, B., Salesse, R., Bidart, J. M., Houllier, A., Guiochon-Mantel, A., Garnier, J., and Milgrom, E. (1990) *Endocrinology 127*, 2090−2098.
6. Ji, I., and Ji, T. H. (1991) *Endocrinology 128*, 2648−2650.
7. Lin, Z., Shenker, A., and Pearlstein, R. (1997) *Protein Eng. 10*, 501−510.
8. Hoflack, J., Hibert, M. F., Trumpp-Kallmeyer, S., and Bidart, J. M. (1993) *Drug Des. Discovery 10*, 157−171.
9. Menon, K. M. J., and Gunaga, K. P. (1974) *Fertil. Steril. 25*, 732−750.
10. Munshi, U. M., Peegel, H., and Menon, K. M. J. (2001) *Eur. J. Biochem. 268*, 1631−1639.
11. Davis, J. S., Weakland, L. L., Farese, R. V., and West, L. A. (1987) *J. Biol. Chem. 262*, 8515−8521.
12. Gudermann, T., Nichols, C., Levy, F. O., Birnbaumer, M., and Birnbaumer, L. (1992) *Mol. Endocrinol. 6*, 272−278.
13. Ascoli, M., Fanelli, F., and Segaloff, D. L. (2002) *Endocr. Rev. 23*, 141−174.
14. Farrens, D. L., Altenbach, C., Yang, K., Hubbell, W. L., and Khorana, H. G. (1996) *Science 274*, 768−770.
15. Ghanouni, P., Steenhuis, J. J., Farrens, D. L., and Kobilka, B. K. (2001) *Proc. Natl. Acad. Sci. U.S.A. 98*, 5997−6002.
16. Javitch, J. A., Fu, D., Liapakis, G., and Chen, J. (1997) *J. Biol. Chem. 272*, 18546−18549.
17. Gether, U., Lin, S., Ghanouni, P., Ballesteros, J. A., Weinstein, H., and Kobilka, B. K. (1997) *EMBO J. 16*, 6737−6747.
18. Rasmussen, S. G., Jensen, A. D., Liapakis, G., Ghanouni, P., Javitch, J. A., and Gether, U. (1999) *Mol. Pharmacol. 56*, 175−184.
19. Dunham, T. D., and Farrens, D. L. (1999) *J. Biol. Chem. 274*, 1683−1690.
20. Shieh, T., Han, M., Sakmar, T. P., and Smith, S. O. (1997) *J. Mol. Biol. 269*, 373−384.
21. Fanelli, F. (2000) *J. Mol. Biol. 296*, 1333−1351.
22. Laue, L. L., Wu, S. M., Kudo, M., Bourdony, C. J., Cutler, G. B., Jr., Hsueh, A. J., and Chan, W. Y. (1996) *Mol. Endocrinol. 10*, 987−997.
23. Kawate, N., Kletter, G. B., Wilson, B. E., Netzloff, M. L., and Menon, K. M. J. (1995) *J. Med. Genet. 32*, 553−554.
24. Robinson, P. R., Cohen, G. B., Zhukovsky, E. A., and Oprian, D. D. (1992) *Neuron 9*, 719−725.
25. Rao, V. R., Cohen, G. B., and Oprian, D. D. (1994) *Nature 367*, 639−642.
26. Paschke, R., Tonacchera, M., Van Sande, J., Parma, J., and Vassart, G. (1994) *J. Clin. Endocrinol. Metab. 79*, 1785−1789.
27. Quintana, J., Wang, H., and Ascoli, M. (1993) *Mol. Endocrinol. 7*, 767−775.
28. Cetani, F., Tonacchera, M., and Vassart, G. (1996) *FEBS Lett. 378*, 27−31.
29. Seifert, R., and Wenzel-Seifert, K. (2001) *Recept. Channels 7*, 357−369.
30. Selley, D. E., Cao, C. C., Liu, Q., and Childers, S. R. (2000) *Br. J. Pharmacol. 130*, 987−996.
31. Ceresa, B. P., and Limbird, L. E. (1994) *J. Biol. Chem. 269*, 29557−29564.
32. Martin, S., Botto, J. M., Vincent, J. P., and Mazella, J. (1999) *Mol. Pharmacol. 55*, 210−215.
33. Neve, K. A., Cumbay, M. G., Thompson, K. R., Yang, R., Buck, D. C., Watts, V. J., DuRand, C. J., and Teeter, M. M. (2001) *Mol. Pharmacol. 60*, 373−381.
34. Glusker, J. P. (1991) *Adv. Protein Chem. 42*, 1−76.
35. Palczewski, K., Kumasaka, T., Hori, T., Behnke, C. A., Motoshima, H., Fox, B. A., Le Trong, I., Teller, D. C., Okada, T., Stenkamp, R. E., Yamamoto, M., and Miyano, M. (2000) *Science 289*, 739−745.
36. Angelova, K., Narayan, P., Simon, J. P., and Puett, D. (2000) *Mol. Endocrinol. 14*, 459−471.
37. Ji, I., and Ji, T. H. (1991) *J. Biol. Chem. 266*, 14953−14957.
38. Lu, Z. L., and Hulme, E. C. (1999) *J. Biol. Chem. 274*, 7309−7315.
39. Shinozaki, H., Fanelli, F., Liu, X., Jaquette, J., Nakamura, K., and Segaloff, D. L. (2001) *Mol. Endocrinol. 15*, 972−984.
40. Fernandez, L. M., and Puett, D. (1996) *Biochemistry 35*, 3986−3993.
41. Jaquette, J., and Segaloff, D. L. (1997) *Endocrinology 138*, 85−91.
42. Dhanwada, K. R., Vijapurkar, U., and Ascoli, M. (1996) *Mol. Endocrinol. 10*, 544−554.
43. Govaerts, C., Lefort, A., Costagliola, S., Wodak, S. J., Ballesteros, J. A., Van Sande, J., Pardo, L., and Vassart, G. (2001) *J. Biol. Chem. 276*, 22991−22999.
44. Kawate, N., Peegel, H., and Menon, K. M. J. (1997) *Mol. Cell. Endocrinol. 127*, 211−219.
45. Horn, F., Vriend, G., and Cohen, F. E. (2001) *Nucleic Acids Res. 29*, 346−349.
46. Berman, H. M., Westbrook, J., Feng, Z., Gilliland, G., Bhat, T. N., Weissig, H., Shindyalov, I. N., and Bourne, P. E. (2000) *Nucleic Acids Res. 28*, 235−242.

47. Pogozheva, I. D., Lomize, A. L., and Mosberg, H. I. (1998) *Biophys. J. 75*, 612−634.
48. Guntert, P., Braun, W., and Wuthrich, K. (1991) *J. Mol. Biol. 217*, 517−530.
49. Yang, K., Farrens, D. L., Hubbell, W. L., and Khorana, H. G. (1996) *Biochemistry 35*, 12464−12469.
50. Altenbach, C., Yang, K., Farrens, D. L., Farahbakhsh, Z. T., Khorana, H. G., and Hubbell, W. L. (1996) *Biochemistry 35*, 12470−12478.
51. Cai, K., Klein-Seetharaman, J., Altenbach, C., Hubbell, W. L., and Khorana, H. G. (2001) *Biochemistry 40*, 12479−12485.
52. Bradbury, F. A., and Menon, K. M. J. (1999) *Biochemistry 38*, 8703−8712.
53. Buettner, K., and Ascoli, M. (1984) *J. Biol. Chem. 259*, 15078−15084.
54. Muller, J., Gondos, B., Kosugi, S., Mori, T., and Shenker, A. (1998) *J. Med. Genet. 35*, 340−341.
55. Perlman, S., Costa-Neto, C. M., Miyakawa, A. A., Schambye, H. T., Hjorth, S. A., Paiva, A. C., Rivero, R. A., Greenlee, W. J., and Schwartz, T. W. (1997) *Mol. Pharmacol. 51*, 301−311.
56. Baranski, T. J., Herzmark, P., Lichtarge, O., Gerber, B. O., Trueheart, J., Meng, E. C., Iiri, T., Sheikh, S. P., and Bourne, H. R. (1999) *J. Biol. Chem. 274*, 15757−15765.
57. Monnot, C., Bihoreau, C., Conchon, S., Curnow, K. M., Corvol, P., and Clauser, E. (1996) *J. Biol. Chem. 271*, 1507−1513.
58. Sheikh, S. P., Vilardarga, J. P., Baranski, T. J., Lichtarge, O., Iiri, T., Meng, E. C., Nissenson, R. A., and Bourne, H. R. (1999) *J. Biol. Chem. 274*, 17033−17041.
59. Bradbury, F. A., Kawate, N., Foster, C. M., and Menon, K. M. J. (1997) *J. Biol. Chem. 272*, 5921−5926.
60. Wilson, M. H., Highfield, H. A., and Limbird, L. E. (2001) *Mol. Pharmacol. 59*, 929−938.
61. Xu, W., Ozdener, F., Li, J. G., Chen, C., de Riel, J. K., Weinstein, H., and Liu-Chen, L. Y. (1999) *FEBS Lett. 447*, 318−324.
62. Williams, A. J., Michel, A. D., Feniuk, W., and Humphrey, P. P. (1997) *Mol. Pharmacol. 51*, 1060−1069.
63. Wenzel-Seifert, K., Hurt, C. M., and Seifert, R. (1998) *J. Biol. Chem. 273*, 24181−24189.

BI026672E