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The Effect of pH on β_2 Adrenoceptor Function

EVIDENCE FOR PROTONATION-DEPENDENT ACTIVATION*

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The transition of rhodopsin from the inactive to the active state is associated with proton uptake at Glu^{134} (1), and recent mutagenesis studies suggest that protonation of the homologous amino acid in the α_{1B} adrenergic receptor (Asp^{142}) may be involved in its mechanism of activation (2). To further explore the role of protonation in G protein-coupled receptor activation, we examined the effects of pH on the rate of ligand-induced conformational change and on receptor-mediated G protein activation for the β_2 adrenergic receptor (β_2AR). The rate of agonist-induced change in the fluorescence of NBD-labeled, purified β_2AR was 2-fold greater at pH 6.5 than at pH 8, even though agonist affinity was lower at pH 6.5. This biophysical analysis was corroborated by functional studies; basal (agonist-independent) activation of Gα_s by the β_2AR was greater at pH 6.5 compared with pH 8.0. Taken together, these results provide evidence that protonation increases basal activity by destabilizing the inactive state of the receptor. In addition, we found that the pH sensitivity of β_2AR activation is not abrogated by mutation of Asp^{130}, which is homologous to the highly conserved acidic amino acids that link protonation to activation of rhodopsin (Glu^{134}) and the α_{1B} adrenergic receptor (Asp^{142}).

A majority of extracellular signaling molecules elicit their physiologic responses through members of the G protein-coupled receptor (GPCR)[1] family. Classically, these extracellular ligands bind and activate their receptors, leading to the stimulation of heterotrimeric GTP-binding proteins (G proteins), which then initiate a cascade of intracellular biochemical changes. Despite diverse physiologic roles that range from neurotransmission to light detection, GPCRs are all believed to share a common seven transmembrane topology, as well as a common activation mechanism. Mutational analyses have assigned ligand binding and G protein coupling to distinct receptor domains (3–5). However, the molecular events responsible for receptor activation remain largely undefined (5).

In an effort to characterize the nature of the structural changes important for GPCR activation, we have applied fluorescent spectroscopic techniques to the β_2 adrenergic receptor (β_2AR), a prototypical member of the seven transmembrane receptor family (6–8). By covalently labeling the receptor with (*N*,*N*′-dimethyl-*N*-(iodoacetyl)-*N*′-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)ethylenediamine) (IANBD), a fluorescent probe that is sensitive to the polarity of its molecular environment, we can directly follow ligand-induced structural dynamics in the receptor. Using this fluorescent spectroscopic method, we recently localized agonist-induced conformational changes to the third and sixth transmembrane (TM) domains of the β_2AR (6). These results are consistent with spectroscopic studies on rhodopsin, which show that movements in TM III and TM VI are involved in its mechanism of activation (9, 10).

The importance of conformational changes in TM III to the mechanism of receptor activation for both rhodopsin and the β_2AR may reflect essential interactions between TM III and agonists. Glu^{113} in TM III of rhodopsin is the counter-ion for the protonated Schiff base formed by retinal and Lys^{296} in TM VII (11), and Asp^{113} in TM III of the β_2AR forms an ion pair with the amine nitrogen of catecholamine agonists (12). Another interesting structural feature of TM III is a highly conserved Glu/Asp-Arg-Tyr (E/DRY) sequence found at the cytoplasmic border of most rhodopsin family GPCRs. Mutagenesis studies in rhodopsin demonstrated that protonation of the glutamic acid in this motif occurs directly after retinal isomerization (1), and mutation to the uncharged glutamine results in a constitutively active opsin (13). Mutagenesis studies and molecular modeling simulations in the α_{1B} adrenergic receptor suggest that activation of the receptor involves conformational changes induced by the protonation of the aspartic acid in this sequence (2). Thus, the agonist-dependent conformational changes we observe in TM III via fluorescence spectroscopic analysis of the β_2AR may reflect this protonation event. Taken together, these studies suggest that a protonation-sensitive conformational state may be important for the mechanism of GPCR activation.

To better understand how protonation influences the activation of a GPCR, we studied the effect of pH on the activity of the β_2AR. We hypothesized that if protonation was important in the mechanism of receptor activation, we would find enhanced

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[1] The abbreviations used are: GPCR, G protein-coupled receptor; TM, transmembrane; IANBD, *N*,*N*′-dimethyl-*N*-(iodoacetyl)-*N*′-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)ethylenediamine; G protein, GTP-binding protein; β_2AR, β_2-adrenergic receptor; NDM, *n*-dodecyl-β-D-maltoside; MOPSO, 3-(*N*-morpholino)-2-hydroxypropanesulfonic acid; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate.

receptor activity at a lower pH. We used fluorescently labeled receptor to study the effect of pH on structural dynamics. In addition, we studied the effect of pH changes on the ability of the receptor to activate its corresponding G protein. The results from these studies provide further evidence for protonation in the process of GPCR activation.

EXPERIMENTAL PROCEDURES

Expression and Purification Procedure of Receptor for Binding Assays and Fluorescence Labeling—DNA sequences encoding the human β_2 adrenergic receptor, epitope-tagged at the amino terminus with the cleavable influenza-hemagglutinin signal sequence followed by the FLAG epitope (IBI, New Haven, CT) and tagged at the carboxyl terminus with six histidines, were cloned into the baculovirus expression vector pVL1392 (Invitrogen, San Diego, CA) (14). This construct was co-transfected with linearized BaculoGold DNA into Sf9 insect cells using the BaculoGold transfection kit (PharMingen, San Diego, CA). The resulting virus was harvested after 4–7 days and amplified once before plaque purification. The plaque-purified viruses were amplified several times to obtain 500 ml of a high titer virus stock (about 1×10^9 plaque-forming units). For purification, the cells were grown in 1000-ml cultures in SF 900 II medium (Life Technologies, Inc.) supplemented with 5% (v/v) fetal calf serum (Gemini, Calabasas, CA) and 0.1 mg/ml gentamicin (Roche Molecular Biochemicals, Mannheim, Germany). Cells were infected with a 1:30–1:40 dilution of a high titer virus stock at a density of 3.5–5.5 $\times$ 10^6 cells/ml and harvested after 48 h by centrifugation (10 min at 5,000 $\times$ *g*). The cell pellets are kept at −70 °C until used for purification. Receptor was purified using a two-step purification procedure. One to two pellets of Sf9 cells from 1000 ml of infected cultures were resuspended in lysis buffer (10 mM Tris-HCl, pH 7.4, with 1 mM EDTA, 10 μg/ml benzamidine (Sigma), 10 μg/ml leupeptin (Roche Molecular Biochemicals), and 0.2 mM phenylmethylsulfonyl fluoride (Sigma)). Following centrifugation (20 min at 30,000 $\times$ *g*), the lysed cells were resuspended in solubilization buffer (20 mM Tris, pH 7.4, with 1.0% *n*-dodecyl-β-D-maltoside (NDM) (Anatrace), 500 mM NaCl, 10 μg/ml benzamidine, 10 μg/ml leupeptin, 0.2 mM phenylmethylsulfonyl fluoride, and 10 μM alprenolol (Sigma)), subjected to 25 strokes from a Dounce homogenizer, and then stirred for 2 h at 4 °C. The solubilized receptor was purified by nickel column chromatography using Chelating Sepharose (Amersham Pharmacia Biotech, Uppsala, Sweden) and alprenolol affinity chromatography as described (8, 14). Approximately 5 nmol of pure protein generally could be obtained from a 1000-ml culture. The specific activity for the purified receptor was ~10 nmol/mg protein. Protein levels were measured with the detergent-insensitive Bio-Rad DC protein assay kit. Purified protein was analyzed by 10% SDS-polyacrylamide gel electrophoresis. The protein was visualized by standard Coomassie staining.

Mutagenesis—The cDNA encoding the human β_2 adrenoreceptor, epitope-tagged at the amino terminus with the cleavable influenza hemagglutinin signal sequence followed by the FLAG epitope (IBI) (15), and tagged at the carboxyl terminus with six histidines (SF-h β_2-6H) was used as the template for mutagenesis (15). The aspartate and glutamate mutations were all generated by polymerase chain reaction-based mutagenesis using *Pfu* polymerase according to the manufacturer's instructions (Stratagene, La Jolla, CA). The generated polymerase chain reaction fragments were digested with the appropriate enzymes, purified by agarose gel electrophoresis, and cloned into the baculovirus expression vector pVL 1392 containing SF-h β_2 -6H fused to Gα_s, constructed as described (16). All mutations were confirmed by restriction enzyme analysis and sequenced.

Membrane Preparation—For infection, cells were sedimented and suspended in fresh medium. Cells were seeded at 3.0 $\times$ 10^6 cells/ml, infected with the optimal dilution of β_2-Gα_s virus stock, as determined by titration, and cultured for 48 h. All membrane preparation steps were done at 4 °C. Cells were harvested by centrifugation (10 min at 10,000 $\times$ *g*), washed once with phosphate-buffered saline and recentrifuged, then resuspended in lysis buffer and lysed using 25 strokes of a Dounce homogenizer. Nuclei and unbroken cells were removed by centrifugation (5 min at 500 $\times$ *g*). The supernatant was removed and centrifuged (30 min at 40,000 $\times$ *g*). The resultant pellet was resuspended in 20 ml of lysis buffer and recentrifuged. Membranes were resuspended at 0.5–1.5 mg of protein/ml in binding buffer (75 mM Tris-HCl, pH 7.4, with 12.5 mM $MgCl_2$, and 1 mM EDTA) and stored at −80 °C until use.

Fluorescent Labeling—Purified receptor was bound to a 100-μl nickel column (Chelating Sepharose). Labeling with the cysteine-reactive fluorophore IANBD (Molecular Probes, Eugene, OR) was achieved by recycling 800 μl of 500 μM IANBD in HS buffer (20 mM Tris-HCl, pH 7.4, containing 500 mM NaCl, and 0.08% NDM) for 30 min. Excess dye was removed by washing with 75 column volumes of HS buffer. Labeled receptor was eluted with 200 mM imidazole in HS buffer. This procedure results in ~1 mol of IANBD/mol of receptor, as determined by measuring absorption at 481 nm and using an extinction coefficient of 21,000 M^{-1} cm^{-1} for IANBD and a molecular mass of 50 kDa for the receptor. Derivatization of the receptor does not perturb receptor pharmacology, and labeling could be selectively blocked by sulfhydryl-reactive agents. (8).

Fluorescence Assays—Spectroscopy was performed on a SPEX Fluoromax spectrofluorometer as described (8). For experiments examining pH affects on the kinetics of the agonist-induced change in fluorescence, 25–50 pmol of IANBD-labeled β_2 receptor were diluted into a solution containing 100 mM NaCl and 1% NDM in a total volume of 475 μl. The solution was constantly stirred for 10 min. The time scan was started and, after 300 s, 25 μl of either 1 M MOPSO, pH 6.5, or 1 M Tris, pH 8.0, were added and allowed to mix for 200 s. (−)-Isoproterenol was then added to a final concentration of 1 mM, and the assay was continued for 2500 s. The time to maximal fluorescence change was calculated by using data from the final 300 s of each experiment (when the fluorescence intensity had stabilized) to extrapolate a line from which all data points after (−)-isoproterenol addition were subtracted. For experiments measuring the effect of pH changes on the magnitude of the agonist-induced fluorescence change, MOPSO (pH 6.5) or Tris (pH 8) were introduced after 200 s and allowed to mix for 100 s. Then (−)-isoproterenol ($[100\ \mu M]_f$) was added and the assay continued for 300 s. At this point, (−)-alprenolol ($[50\ \mu M]_f$) was added and the assay continued until the new base line had stabilized. The magnitude of the isoproterenol-induced fluorescence change was determined by comparing fluorescence intensity, corrected for dilution, before and after (−)-alprenolol addition. These corrected data were subjected to non-linear regression analysis to determine the time at which the half of the maximal change in fluorescence was reached. All of the compounds in the concentrations used had an absorbance of less than 0.01 at 481 nm ($\lambda_{excitation}$) and 523 nm ($\lambda_{emission}$), excluding any inner filter effects.

Binding Assays—Binding assays on purified β_2 receptor were performed using [^{3}H]dihydroalprenolol as radioligand (Amersham Pharmacia Biotech) as described previously (8). On-rates were assessed by incubating purified receptor with 10 nM [^{3}H]dihydroalprenolol in a total volume of 100 μl of binding buffer (100 mM NaCl, 1% NDM, with either 30 mM MOPSO, pH 6.5, or 30 mM Tris-HCl, pH 8) to determine optimal time for subsequent incubations (data not shown). To examine the stability of the purified receptor under conditions of varying pH, the receptor was diluted 1:200 in buffer containing 100 mM NaCl, 1% NDM, and 50 mM amounts of the appropriate buffer. After either 1 or 3 h of incubation, 10 μl of this dilution was used for a 1 h binding assay containing 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1% NDM, and 10 nM [^{3}H]dihydroalprenolol in a total volume of 100 μl. For saturation binding assays, purified and solubilized receptor was incubated for 30 min in 100 μl of binding buffer containing [^{3}H]dihydroalprenolol varying in concentration from 22 to 0.69 nM. In competition binding assays, purified receptor was incubated for 30 min in 100 μl of binding buffer containing either (−)-alprenolol or (−)-isoproterenol (from 10^{-4} to 10^{-9} M). The (−)-isoproterenol competition assays contained 1.4 nM [^{3}H]dihydroalprenolol for experiments at pH 8 and 5.5 nM [^{3}H]dihydroalprenolol for pH 6.5 experiments, while the (−)-alprenolol competition assays contained 2.1 nM [^{3}H]dihydroalprenolol for experiments at pH 8 and 10.3 nM [^{3}H]dihydroalprenolol for experiments at pH 6.5. We were unable to consistently perform saturation binding on the purified receptor at pH 6.5. We therefore determined the dissociation constant (K_D) at pH 6.5 by competing [^{3}H]dihydroalprenolol with alprenolol. We determined the K_D at pH 8.0 by both saturation and competition methods; these methods yielded the same value, thus confirming the validity of the competition assay approach for determining the dissociation constant at pH 6.5. With the goal of obtaining approximately 10% bound radioligand, both saturation and competition binding assays conducted at pH 6.5 contained between 4 and 6 times the amount of purified receptor as those experiments performed at pH 8.0. All binding assays were stopped and free [^{3}H]dihydroalprenolol separated from bound by desalting on Sephadex G50 columns (4 cm $\times$ 0.5 cm) using ice-cold elution buffer (20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 0.08% NDM). All nonspecific binding was determined in the presence of 10 μM alprenolol where appropriate. For saturation binding experiments on the β_2-Gα_s constructs, Sf9 membranes (10–40 μg of protein) were suspended in 500 μl of binding buffer supplemented with 0.1–10 nM [^{3}H]dihydroalprenolol and 0.2% (w/v) bovine serum albumin. Nonspecific binding was assessed in the presence of 10 μM (−)-alprenolol. Incubations were performed for 1 h at 25 °C with shaking at 200 rpm. Competition binding experiments were carried out with 1.2 nM [^{3}H]dihydroalpreno-

lol in the presence of increasing concentrations of (−)-isoproterenol. Binding data were analyzed by nonlinear regression analysis using Prism 2.0 (GraphPad Software, San Diego, CA). K_I was calculated from the equation $K_I = IC_{50}/(1 + L/K_D)$.

Measurement of GTPase Activity—Before experiments, membranes were thawed at 4 °C, centrifuged (15 min at 15,000 × *g*) and resuspended in 10 mM Tris, pH 7.4. Assay tubes contained β_2-Gα_s or Gα_s membranes (10 μg of protein), 100 nM GTP, 1 mM $MgCl_2$, 100 μM EDTA, 100 μM ATP, 1 mM adenylyl imidodiphosphate, 5 mM creatine phosphate, 40 μg of creatine kinase, and 0.2% (w/v) bovine serum albumin in 50 mM MOPSO, pH 6.5, or 50 mM Tris-HCl, pH 8.0. Tubes also contained ligands at various concentrations. Assay tubes (80 μl) were incubated for 3 min at 25 °C before the addition of 20 μl of [γ-^{32}P]GTP (0.1–0.5 μCi/tube). Reactions were conducted for 20 min at 25 °C and were terminated by the addition of 900 μl of a slurry consisting of 5% (w/v) activated charcoal in 50 mM NaH_2PO_4, pH 2.0. Reaction mixtures were centrifuged (15 min at 15,000 × *g*) at room temperature. 700 μl of the supernatant were removed, and [^{32}P]P_i was measured by liquid scintillation counting.

RESULTS

Effect of pH on β_2AR Stability and Ligand Affinity—The β_2AR contains 34 acidic amino acids (Asp and Glu) and 45 basic amino acids (Lys, Arg, and His). In addition to their potential involvement in the process of receptor activation, these pH-sensitive amino acids may be involved in maintaining the tertiary structure of the receptor. We therefore examined the pH range over which the β_2AR remains stable as determined by binding to the antagonist [^{3}H]dihydroalprenolol. The results shown in Fig. 1*A* demonstrate that radioligand binding can be measured over a broad pH range from 7.0 to 9.0. There is a dramatic loss of receptor activity below pH 7, with nearly complete loss of binding at pH 6. Based on these studies, we chose to focus on a comparison of receptor at pH 8.0, where the receptor appeared most stable, and pH 6.5, where we began to observe a loss of function, suggesting that one or more of the acidic amino acids were being protonated.

The loss of radioligand binding observed in Fig. 1*A* could be due to denaturation of the receptor and/or to a more specific effect of pH on ligand binding. To assess receptor stability, the pH of purified receptor was adjusted to either pH 6.5 or 8.0. After a 1- or 3-h incubation at room temperature, ligand binding was performed at pH 8 with a saturating (10 nM) concentration of [^{3}H]dihydroalprenolol to determine the recovery of functional receptor. As shown in Fig. 1*B*, there was a significantly greater loss of binding activity in receptor incubated at pH 6.5.

The isoproterenol (agonist) and dihydroalprenolol (antagonist) binding affinity for purified, detergent-solubilized β_2AR receptor at pH 6.5 and pH 8.0 are presented in Table I. The affinities for both agonist and antagonist were reduced by approximately 10-fold at pH 6.5.

Effect of pH on Agonist-induced Conformational Changes—We have previously shown that fluorescent techniques can be used to monitor ligand-induced conformational changes in the β_2 adrenergic receptor. Purified, detergent-solubilized β_2AR was labeled with IANBD, a fluorescent probe that is sensitive to the polarity of its molecular environment. We then examined agonist-induced changes in receptor fluorescence at pH 8.0 and at pH 6.5 (Fig. 2*A*). The rate of conformational change, as reflected by the rate of the agonist-induced fluorescence change, was 2.1 times faster at pH 6.5 ($t_{1/2}$ = 186 ± 10 s) than at pH 8.0 ($t_{1/2}$ = 391 ± 48 s). The increased rate of fluorescence change at pH 6.5 could be due to an increase in the rate of agonist-induced conformational change or to protein denaturation, since we noted greater instability at pH 6.5. To distinguish between these two possibilities, we looked at the ability of antagonists to reverse the changes in fluorescence. (−)-Alprenolol rapidly reverses agonist-induced changes in fluorescence; however, exposure of receptor to (−)-alprenolol alone has no effect on receptor fluorescence (8). Thus, (−)-alprenolol would not be expected to reverse conformational changes due to protein denaturation. However, if conformational changes occur more rapidly at pH 6.5 than at pH 8.0, we would expect that the magnitude of the (−)-alprenolol reversal after a short exposure to isoproterenol would be greater at pH 6.5 than at pH 8.0. Therefore, we examined the ability of (−)-alprenolol to reverse the conformational change induced by exposure to isoproterenol (Fig. 2*B*). We found that that the extent of reversal after the addition of the antagonist was significantly greater at pH 6.5 (4.5 ± 0.2%) than that at pH 8.0 (3.5 ± 0.2%). Thus the increased rate of agonist-induced fluorescence at pH 6.5 can be attributed to an increase in the rate of reversible, agonist-induced conformational changes in the receptor.

It is well known that the intensity of fluorescent molecules can be influenced by pH. However, NBD is relatively pH-insensitive, and we examined agonist-induced changes in fluorescence at a given pH in each experiment, so that the pH was not changed while fluorescence was being monitored. Thus, the effect of pH on the rate of agonist-induced conformational change cannot be explained by pH-sensitive properties of NBD.

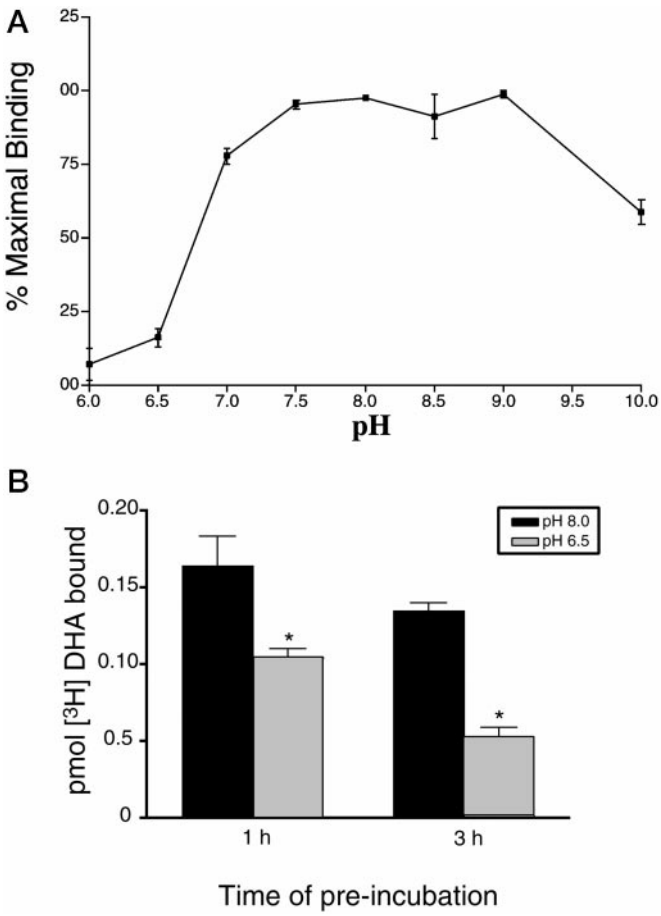

FIG. 1. *A*, measurement of receptor binding across a broad pH range. Purified β_2AR was diluted 1:300 into 100 mM NaCl, 1% *n*-dodecyl maltoside. Saturation binding was performed in 30 mM amounts of the appropriate buffers, spanning the range from pH 6 to pH 10. Data represent triplicate values. *B*, incubation at pH 6.5 causes loss of receptor antagonist binding. Purified β_2AR was incubated for the indicated times with buffer containing either 20 mM MOPSO, pH 6.5, or 20 mM Tris, pH 8.0. Radioligand binding was then assayed in a 20 mM Tris, pH 8.0, buffer. Binding after pre-incubation for 1 h at pH 8 and pH 6.5 was 0.16 ± 0.02 pmol of [^{3}H]dihydroalprenolol and 0.10 ± 0.01 pmol of [^{3}H]dihydroalprenolol, respectively (p = 0.04 in a two-tailed, unpaired t test). Binding after pre-incubation for 3 h at pH 8 and 6.5 was 0.13 ± 0.006 pmol of [^{3}H]dihydroalprenolol and 0.053 ± 0.006 pmol of [^{3}H]dihydroalprenolol, respectively (p = 0.0006 in a two-tailed, unpaired t test; all data represent mean ± S.E., n = 3).

TABLE I
Effect of pH on agonist and antagonist binding affinities

K_D was determined at pH 8.0 via saturation and competition assays, both methods yielding the same value. The competition method was used for pH 6.5. ALP is the neutral antagonist (−)-alprenolol, and ISO is the full agonist (−)-isoproterenol.

	K_D (ALP)	K_I (ISO)
	nM	*μM*
pH 6.5	35 ± 3 (n = 4)	4.6 ± 0.4 (n = 3)
pH 8.0	2.0 ± 0.3 (n = 4)	0.56 ± 0.13 (n = 4)

Effect of pH on GTPase Activity in the β_2-Gα_s Fusion Protein—To examine the influence of pH on interactions between the β_2AR and its preferred G protein, Gα_s, we monitored ligand-regulated GTPase activity. However, even when the β_2AR is expressed with a large excess of Gα_s in insect cells, the efficiency of coupling is not sufficient to permit the use of a GTPase assay to directly monitor interactions between receptor and G protein (16). Therefore, we used a fusion protein between the β_2AR and Gα_s (β_2-Gα_s). Others and we have observed that fusion of the β_2AR with Gα_s leads to highly efficient coupling and permits the direct assessment of Gα_s activation by techniques such as GTPase activity and GTPγS binding (16–19).

We examined basal and ligand-modulated GTPase activity in Sf9 membranes expressing β_2-Gα_s. As shown in Fig. 3, basal GTPase activity was 1.7 times higher at pH 6.5 than at pH 8.0 (2.6 ± 0.2 pmol of ^{32}P released mg^{-1} min^{-1} *versus* 1.5 ± 0.2 pmol of ^{32}P released mg^{-1} min^{-1}). In contrast, the maximal GTPase activation by the agonist isoproterenol (*ISO*) was lower at pH 6.5. As another measure of the constitutive receptor activity, we also examined the proportion of the basal activity that was inhibited by the inverse agonist ICI 118,551 (*ICI*). At pH 8.0, the inverse agonist inhibited 7.9 ± 2.8% of ligand-regulated activity, whereas at pH 6.5, ICI inhibited 37.6 ± 2.5% of ligand-regulated activity; the higher efficacy of ICI at pH 6.5 indicates a higher proportion of basal activity at that pH. These results also serve to confirm that the higher basal GTPase activity at pH 6.5 was mediated by the receptor and not by a direct effect of pH on Gα_s. To further investigate the effect of pH on Gα_s, we examined Sf9 membranes expressing an equivalent amount of Gα_s alone (as determined by Western blot analysis). In these membranes, there was no significant difference in basal GTPase activity at pH 6.5 compared with the activity at pH 8.0 (Fig. 3).

In an attempt to identify the site or sites responsible for pH-dependent effect on basal activity, we generated β_2-Gα_s fusion proteins in which specific acidic amino acids in the β_2AR were mutated to alanine. Glu^{122}, Asp^{130}, and Glu^{268} were chosen, based on predictions made from a three-dimensional model of the β_2AR (see "Discussion"). The positions of these mutations in the predicted secondary structure of the β_2AR are shown in Fig. 4*A*. All three mutated receptors were expressed in Sf9 cells, and membranes were prepared for ligand binding and GTPase studies. As a measure of constitutive activity, we examined the effect of the inverse agonist ICI on basal activity and expressed this as a percentage of the total ligand-regulated GTPase activity (100 × [basal − ICI_{MAX})/(ISO_{MAX} − ICI_{MAX}]). All of the mutated receptors showed increased basal activity at pH 6.5 compared with pH 8.0, as determined by the enhanced effect of ICI at pH 6.5 (Fig. 4*B*). The pharmacological properties of these mutants are given in Table II. All three mutant β_2-Gα_s fusion proteins were indistinguishable from the wild type fusion protein with respect to the fraction and the K_I of high affinity binding sites and to the EC_{50} for agonist stimulation of GTPase activity. The K_I of the low affinity site for the E122A fusion protein was significantly higher than that of the wild type and the other mutant fusion proteins. All three mutants had a slightly reduced affinity for the antagonist dihydroalprenolol compared with the wild type β_2-Gα_s fusion protein.

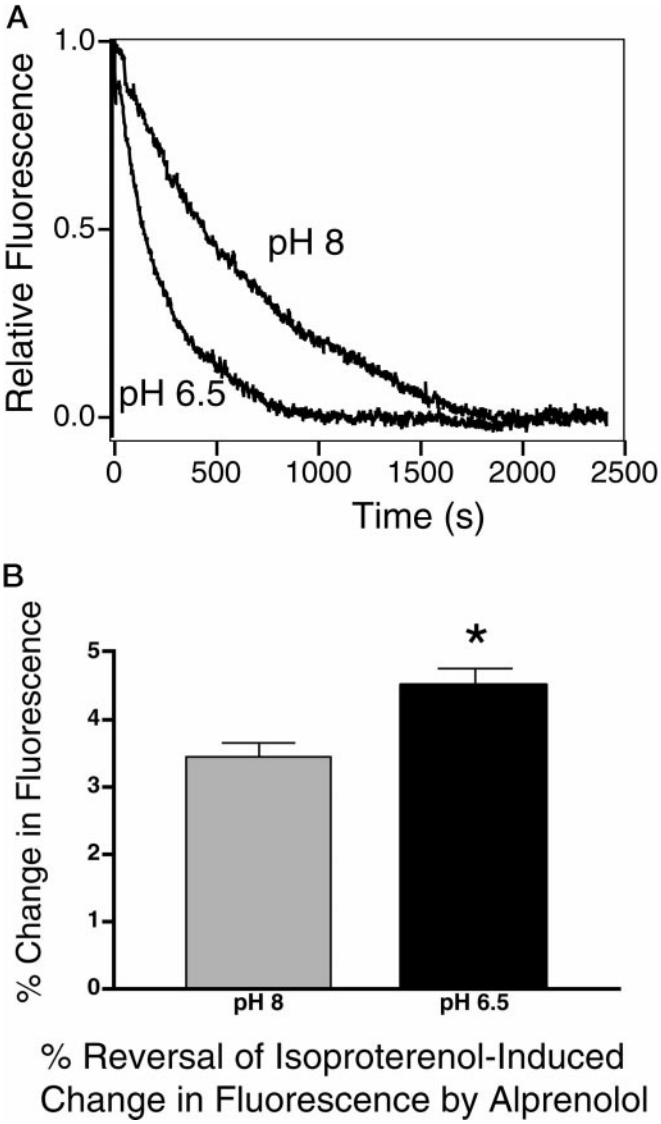

FIG. 2. **Effect of pH change on the rate of agonist-induced receptor conformational change.** *A*, β_2AR was purified and labeled with the environmentally sensitive fluorescent probe IANBD as described under "Experimental Procedures." The change in fluorescence intensity over time served as a measure of the agonist-induced conformational changes in the receptor. The rate of the fluorescence change subsequent to (−)-isoproterenol exposure varied depending on the pH. At pH 8.0, the $t_{1/2}$ for the maximal fluorescence change was 391 ± 48 s, whereas at pH 6.5, the $t_{1/2}$ for the maximal fluorescence change was 186 ± 10 s (p = 0.002 in a two-tailed, unpaired t test, n = 6). These curves are representative of three experiments in which purified, NBD-labeled receptor was diluted into buffers at either pH 6.5 or pH 8.0, and then exposed to a saturating concentration (1 mM) of the agonist (−)-isoproterenol. Fluorescence change was monitored for 2500 s in each experiment. *B*, reversal of agonist-induced fluorescence changes by the antagonist (−)-alprenolol. IANBD-labeled receptor was incubated for 5 min with 100 μM isoproterenol. We then monitored the increase in fluorescence intensity that occurred after the addition of 10 μM (−)-alprenolol. At pH 8.0, the reversal was 3.5 ± 0.2%, while at pH 6.5, the reversal was 4.5 ± 0.2% (p = 0.0065 in a two-tailed, unpaired t test, n = 6). Data analysis is described under "Experimental Procedures."

DISCUSSION

According to the extended ternary complex model, which is commonly used in the discussion of receptor activation, the receptor exists in an equilibrium between two states: the inactive state (R) and the active state (R*) (20). Agonists stabilize

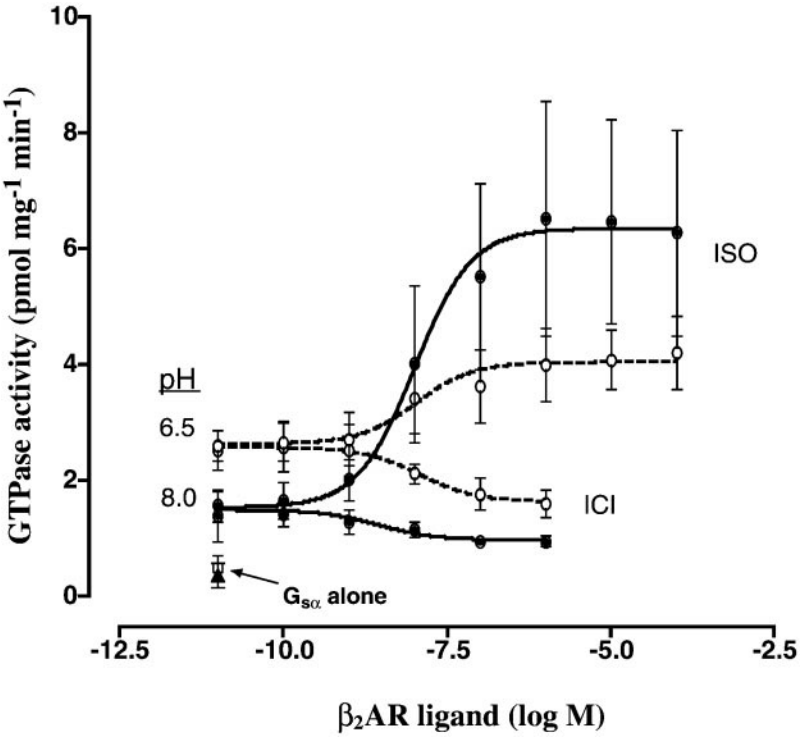

FIG. 3. **Effect of pH on receptor-regulated GTPase activity.** Sf9 cell membranes expressing β_2-Gα_s fusion protein were used to monitor GTP hydrolysis at pH 8.0 (●) and pH 6.5 (○). Receptor-dependent GTPase activity was stimulated by the full agonist (−)-isoproterenol (*ISO*) and inhibited by the inverse agonist ICI-118,551 (*ICI*). Basal activity at pH 6.5 was significantly elevated relative to pH 8.0 (2.6 ± 0.2 pmol of ^{32}P released mg^{-1} min^{-1} *versus* 1.5 ± 0.2 pmol of ^{32}P released mg^{-1} min^{-1}, $p = 0.005$ in a two-tailed, unpaired t test, $n = 6$). The total activity was defined as the range of activity at the maximal ligand concentrations and the proportion of the total activity that was inhibited by ICI-118,551 was thus calculated from (basal $-ICI_{MAX}$)/($ISO_{MAX} - ICI_{MAX}$). The proportion of the total activity that was inhibited by ICI-118,551 was 37.6 ± 2.5% at pH 6.5 and 7.9 ± 2.8% at pH 8.0 ($p = 0.001$ in a two-tailed, unpaired t test, $n = 3$). The effect of pH on Gα_s-expressing membranes is also indicated on the graph to allow comparison with the receptor-stimulated basal activities. There was no significant difference in these values (pH 8.0 (▲) = 0.375 ± 0.21 pmol of ^{32}P released mg^{-1} min^{-1} *versus* pH 6.5 (□) = 0.512 ± 0.22 pmol of ^{32}P released mg^{-1} min^{-1}; $p = 0.57$ in a two-tailed, unpaired t test, $n = 3$). The membranes used for these assays expressed β_2-Gα_s fusion proteins at 7.5 pmol/mg.

R*, whereas inverse agonists stabilize R. In the absence of agonist, the small proportion of receptor in R* is responsible for the basal activity observed for many GPCRs. It has been proposed that mutations that increase basal activity, also called constitutively activating mutations, do so by favoring the transition from R to R*. Other properties commonly associated with receptors having constitutively activating mutations include an increased agonist affinity and an increased efficacy of inverse agonists, both properties attributable to the higher proportion of receptors in R* (2, 20, 21). While this simple two-state model may not be able to explain all properties of GPCR activation (22), we will use it as a point of reference to discuss our studies on the effect of pH on β_2AR activation.

To examine the effect of pH on β_2AR activation, we first examined the pH range over which the receptor is stable. The loss of binding activity that we observed between pH 6 and 7 can be explained by the effect of pH on ligand binding affinity and on protein stability. The reduction in agonist and antagonist binding affinity at pH 6.5 (Table I) may be due to protonation of Asp^{113} in TM III, which is thought to serve as a counter-ion for the amine nitrogen of catecholamine agonists and antagonists (12). It is also possible that the reduced binding affinity is due to some structural change that results from protonation of other acidic amino acids.

Stability studies on purified β_2AR also show an increased rate of denaturation at pH 6.5 relative to pH 8 (Fig. 1*B*). Protonation of acidic amino acids not directly involved in binding or receptor activation may cause denaturation of the receptor at reduced pH. However, it is interesting to consider the effects of pH 6.5 on the β_2AR (reduced stability and increased basal activity) in light of our previous observations of the molecular and structural characteristics of a constitutively active mutant of the β_2 adrenergic receptor. We found that the enhanced basal activity of the constitutively active mutant receptor was associated with structural instability in the receptor (7). It is possible that intramolecular interactions that maintain the receptor in an inactive conformation also prevent denaturation of the protein. These intramolecular interactions may be lost by activating mutations or protonation of acidic amino acids involve in intramolecular salt bridges.

We have previously demonstrated that fluorescence spectroscopy can be used to follow ligand-induced conformational changes in purified, detergent-solubilized β_2AR labeled with the cysteine-reactive fluorescent probe IANBD. Using this approach, we analyzed the effect of pH on the kinetics of the agonist-induced conformational change. We observed a greater than 2-fold increase in the rate of agonist-dependent conformational change in the receptor at pH 6.5 in comparison with pH 8.0. This result is consistent with the presence of a pH-sensitive conformational state in the transition from R to R*. The more rapid kinetics of the fluorescence change at pH 6.5 cannot be explained by pH-dependent changes in binding affinity, since we observed a higher K_I for (−)-isoproterenol at pH 6.5. Furthermore, the more rapid agonist-induced change in fluorescence at pH 6.5 cannot be attributed to nonspecific, pH-induced changes in protein structure, since the fluorescence changes were reversed by the neutral antagonist alprenolol (Fig. 2*B*).

Our finding that lowering the pH increases the rate of agonist-induced conformational change is consistent with the hypothesis that acidification, and thus presumably protonation of one or more amino acids, favors the transition from R to R*. Our results with the β_2-Gα_s fusion protein most clearly dem-

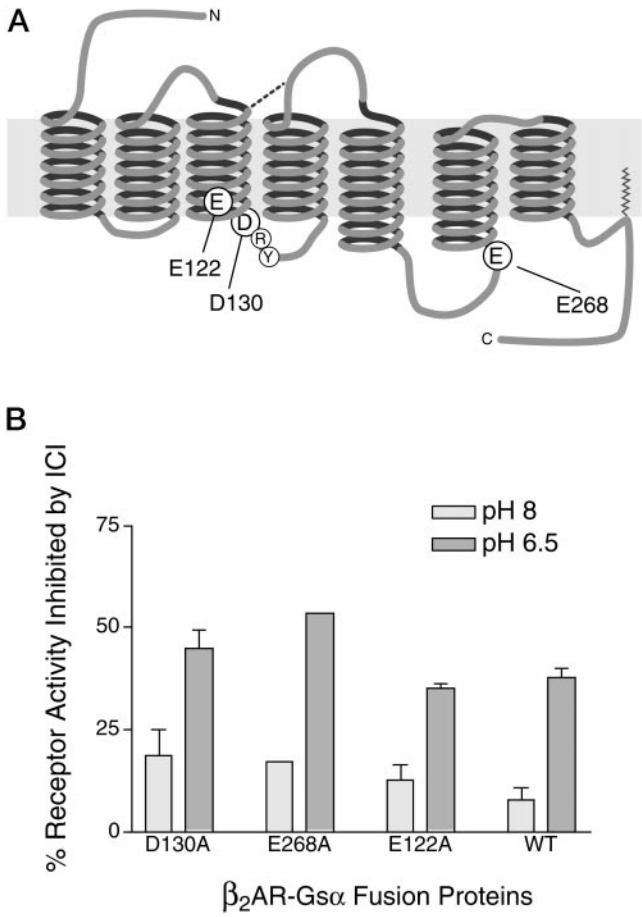

FIG. 4. *A*, positions of potential pH-regulated sites mutated in the β_2-Gα_s fusion proteins. The indicated amino acids (*large circles*) were chosen based on predictions made from a three-dimensional model of the β_2AR (see "Discussion"). *B*, effect of pH on GTPase activity of mutant β_2 receptors. Sf9 cell membranes expressing β_2-Gα_s fusion proteins mutated at the indicated acidic residues were used to monitor GTP hydrolysis at pH 8.0 and 6.5. Receptor-dependent GTPase activity was stimulated by the full agonist (−)-isoproterenol (*ISO*) and inhibited by the inverse agonist ICI-118,551 (*ICI*). The proportion of the total activity that was inhibited by ICI-118,551 was calculated as in Fig. 3. All values represent the average of three experiments done in duplicate, except for E268A, which is the average of two experiments done in duplicate, the values for which are 18.6 and 15.9% at pH 8 and 54.4 and 52.3% at pH 6.5. The membranes used for these assays expressed β_2-Gα_s fusion proteins at 2–4 pmol/mg.

onstrated an increase in the level of basal, agonist-independent receptor activity as pH was lowered. We noted that basal activity was approximately 2-fold greater at pH 6.5 compared with the activity at pH 8. Thus, the lower pH favored the formation of the active R* state in the absence of agonist. The heightened responsiveness to the inverse agonist ICI provides further evidence for a pH-dependent increase in the level of basal receptor activity. As seen in Fig. 3, the response to ICI was nearly 5-fold greater at pH 6.5 than at pH 8. In contrast, we observe a smaller response to the agonist ISO at pH 6.5, perhaps reflecting the lower stability of the β_2AR at pH 6.5. In fact, the elevation in constitutive activity found at pH 6.5 becomes even more impressive, when considering that the number of functional receptors is probably reduced at that pH. Without denaturation, we might expect the level of basal activity at pH 6.5 to be even greater.

While the GTPase studies provide evidence that protonation leads to receptor activation, the kinetic information obtained from the fluorescence studies provides insight into the mechanism of this activation, insight that cannot be deduced from steady state studies such as GTPase or adenylyl cyclase assays.

TABLE II

Pharmacological properties of the β_2-Gα_S fusion proteins

The notation for the fusion proteins indicates the mutation made in the receptor portion of the fusion construct. K_D values were calculated for the neutral antagonist (−)-alprenolol and K_I values for the full agonist (−)-isoproterenol. The two values for K_I represent the two affinity states of the receptor for agonist in the presence of the G protein; in all cases, competition binding isotherms were fit better by a two-site *versus* a single-site curve. $\%R_{high}$ indicates the percentage of high affinity binding sites. EC_{50} values are from the GTPase assay performed at pH 8.0. The values for the affinity of the wild type β_2-Gα_S were taken from reference 19. All other values represent the average of three independent experiments and are reported with S.E., except for the EC_{50} calculation of the E268A fusion protein, which is the average of two experiments. All values were determined in membranes expressing fusion proteins at 2.0–9.0 pmol/mg.

β_2-Gα_S	K_D	$K_{I(low)}$	$K_{I(high)}$	$\%R_{high}$	EC_{50}
	nM	*nM*	*nM*		*nM*
Wild type	0.36 ± 0.3	124 ± 19	3.0 ± 1.3	42.5 ± 7.5	12 ± 4
D130A	1.3 ± 0.1	42 ± 12	1.8 ± 0.8	45.7 ± 4.2	17 ± 4
E268A	1.2 ± 0.3	70 ± 22	1.5 ± 0.5	49.6 ± 4.1	28 ± 2
E122A	1.5 ± 0.2	712 ± 27	5.5 ± 0.6	42.1 ± 1.3	19 ± 9

The greater basal receptor activity observed at reduced pH suggests that protonation favors the transition of the receptor from R to R*. This could due to stabilization of R* or destabilization of R. Stabilization of R* would result in a greater proportion of the receptor in R* at equilibrium, but would not be expected to affect the rate of the agonist-induced transition from R to R*. However destabilization of the R state would be predicted to accelerate the rate of the agonist-induced change. As discussed above, destabilization of R can also explain the more rapid rate of denaturation that we observe at pH 6.5.

It is interesting to compare our results with related studies on rhodopsin. Rhodopsin activation is commonly assessed by following the spectral changes that occur during the conversion between metarhodopsin I (MI) and metarhodopsin II (MII). This MII intermediate is generally accepted as the activated (R*) form of rhodopsin, which is capable of binding transducin and activating guanine nucleotide exchange (11). Previous work has demonstrated a pH dependence of the rate of the MI to MII conversion (23, 24). Consistent with our findings, Parkes and Liebman (23) found that the rate of the light-induced conversion between MI and MII was increased by decreasing the pH from 7.7 to 6.1. A highly conserved sequence among GPCRs at the end of TM III, E/DRY, has been examined as the potential site of this pH dependence. Mutation of the acidic residue in this sequence to mimic a protonation event electrostatically (*i.e.* in rhodopsin, $Glu^{134} \rightarrow Gln$) results in constitutive activation as assessed by the ability of opsin (rhodopsin without retinal) to induce GTPγS binding by transducin (13). Glu^{134} has also been shown to mediate light dependent proton uptake during rhodopsin activation (1). In addition, electron paramagnetic resonance analyses of this $Glu^{134} \rightarrow Gln$ mutant showed that the dark state of this mutant shares conformational similarities with the photoactivated conformations of wild type rhodopsin (25). In the α_{1B} adrenergic receptor, studies found that similar mutation of the analogous Asp^{142} site constitutively activated that receptor in assays of inositol phosphate accumulation, while molecular modeling of the α_{1B} adrenergic receptor suggested that this activation correlated with protonation of Asp^{142} (2).

In light of these results, we attempted to identify the site in the β_2AR that conferred pH dependence to activation by focusing on the analogous site in the conserved E/DRY sequence, Asp^{130}. Using an $Asp^{130} \rightarrow Ala$ β_2-Gα_s fusion protein, we found that the removal of this acidic residue did not ablate the pH-dependent increase in basal activity (Fig. 4*B*). Thus, these results provide evidence that the stimulation of receptor activ-

ity by acid pH is not solely due to proton uptake by the acidic residue in the highly conserved E/DRY sequence at the cytoplasmic face of TM 3.

In an attempt to identify other residues that might be important for the increase in ligand-independent activity noted at pH 6.5, we examined the location of acidic amino acids in a molecular model of the β_2AR based on the Baldwin and Schertler model of rhodopsin (26). Some acidic residues are highly conserved within GPCRs, such as Asp^{79}, Asp^{113}, Asp^{130}, and Glu^{268}, and might be expected to play an important role in receptor structure, complicating interpretation of potential mutagenesis studies. As noted above, Asp^{113} is essential for catecholamine binding and previous studies have shown that mutation of Asp^{79} severely impairs G protein coupling (27), so these sites were excluded from analysis. Non-conserved acidic residues were largely eliminated from further consideration because they appeared to be located on an exposed, highly hydrated surface of the receptor where they would not be expected to influence receptor activity in response to pH changes. However, Glu^{122} is located at the cytoplasmic face of TM 3 and was therefore selected for further analysis. We generated β_2-Gα_s fusion proteins having $Glu^{268} \rightarrow$ Ala or $Glu^{122} \rightarrow$ Ala mutations and examined the effect of pH on basal activity. Neither of these mutations ablated the elevation of basal receptor activity that occurred with the wild type receptor at pH 6.5 (Fig. 4). Thus, it appears that none of these acidic amino acids are, by themselves, responsible for the elevated basal activity at pH 6.5.

These findings corroborate studies on rhodopsin, where mutation of Glu^{134} did not inhibit the stimulation of activation by lowered pH (28). Indeed, other studies in rhodopsin have identified certain conserved histidines as pH-dependent regulators of the formation of the MII activated intermediate (24). It was not possible to study agonist dependent conformational changes in the $Asp^{130} \rightarrow$ Ala, $Glu^{122} \rightarrow$ Ala, or $Glu^{268} \rightarrow$ Ala receptor mutants. These mutants expressed poorly in Sf9 cells, and we were unable to purify sufficient quantities for biophysical studies.

Our biochemical studies are limited by our inability to assess the effect of pH on activation independent of their effects on ligand binding (most likely due to protonation of Asp^{113}). It is interesting to note that in an intact cell, the local pH surrounding Asp^{113}, on the extracellular side of TM III, is probably different from pH surrounding intracellular domains. Asp^{113}, which is critical for ligand binding, faces the extracelluar environment, with a pH of approximately 7.4 *in vivo*, while the pH of the cytosol can range from 6.7 to 7.1 depending on the cell and the method used to determine the pH (29). Thus, the pH gradient across the plasma membrane in an intact cell may facilitate agonist activation of the β_2AR.

In summary, we have investigated the pH dependence of β_2AR activation. Using a biophysical and a biochemical approach, we find evidence that acidification facilitates the transition of the receptor from the inactive to the active state. The activation process may be facilitated by protonation of one or more amino acids that have yet to be identified. The similarity of our findings with earlier studies of rhodopsin suggests that a pH-sensitive conformational change may be common for the formation of the active state of other GPCRs. However, we found that the pH sensitivity of β_2AR activation is not mediated solely by Asp^{130}, which is homologous to the highly conserved acidic amino acids that have been proposed to link protonation to activation of rhodopsin (Glu^{134}) and the α_{1B} adrenergic receptor (Asp^{142}).

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