

## Identification of an Allosteric Binding Site for Zn<sup>2+</sup> on the $\beta_2$ Adrenergic Receptor\*

Received for publication, June 28, 2002, and in revised form, October 28, 2002  
Published, JBC Papers in Press, October 29, 2002, DOI 10.1074/jbc.M206424200

Gayathri Swaminath, Tae Weon Lee, and Brian Kobilka‡

From the Department of Molecular and Cellular Physiology, Howard Hughes Medical Institute, Stanford Medical Center, Stanford, Palo Alto, California 94305

**The activity of G protein-coupled receptors (GPCRs) can be modulated by a diverse spectrum of drugs ranging from full agonists to partial agonists, antagonists, and inverse agonists. The vast majority of these ligands compete with native ligands for binding to orthosteric binding sites. Allosteric ligands have also been described for a number of GPCRs. However, little is known about the mechanism by which these ligands modulate the affinity of receptors for orthosteric ligands. We have previously reported that Zn(II) acts as a positive allosteric modulator of the  $\beta_2$ -adrenergic receptor ( $\beta_2$ AR). To identify the Zn<sup>2+</sup> binding site responsible for the enhancement of agonist affinity in the  $\beta_2$ AR, we mutated histidines located in hydrophilic sequences bridging the seven transmembrane domains. Mutation of His-269 abolished the effect of Zn<sup>2+</sup> on agonist affinity. Mutations of other histidines had no effect on agonist affinity. Further mutagenesis of residues adjacent to His-269 demonstrated that Cys-265 and Glu-225 are also required to achieve the full allosteric effect of Zn<sup>2+</sup> on agonist binding. Our results suggest that bridging of the cytoplasmic extensions of TM5 and TM6 by Zn<sup>2+</sup> facilitates agonist binding. These results are in agreement with recent biophysical studies demonstrating that agonist binding leads to movement of TM6 relative to TM5.**

G protein-coupled receptors (GPCR)<sup>1</sup> respond to a wide range of structurally diverse natural and synthetic ligands. The majority of these ligands are classified as orthosteres. They compete for binding with the natural ligand, and their binding site is predicted to overlap with that of the natural ligand. Allosteric modulation of ligand binding has been observed for several GPCRs. Allosteric modulators include physiologically relevant ions as well as small organic molecules. A large number of compounds allosterically increase binding affinity for muscarinic receptor antagonists. Allosteric regulation can be demonstrated in all five subtypes of muscarinic receptors, but the m2 receptor appears to be the most sensitive (1). Although most

allosteric modulators for the muscarinic receptors affect antagonist binding, allosteric modulation of agonist binding has also been described (2).

In addition to the muscarinic receptor, allosteric compounds have been identified for the A1 adenosine receptor. The 2-amino-3-benzoylthiophene, PD 81,723, has been shown to enhance agonist binding and G protein coupling in a Mg<sup>2+</sup>-dependent manner (3, 4).

The function of several G<sub>i</sub>-coupled receptors has been shown to be modulated by amiloride analogs and by Na<sup>+</sup>. This effect has been particularly well characterized for the  $\alpha_2$  adrenergic receptor (5–7) and for the dopamine D2 (8) and D4 subtypes (9). Na<sup>+</sup> both uncouples the receptor from G<sub>i</sub> and reduces agonist affinity. A conserved Asp residue within the cytoplasmic side of TM2 is responsible for the Na<sup>+</sup>-sensitive binding of the  $\alpha_2$ A receptor (6) and the D4 dopamine receptor (9). This Na<sup>+</sup> sensitivity may be physiologically relevant, as relatively high local concentrations of Na<sup>+</sup> ions may accumulate at the cytoplasmic side of the receptor after membrane depolarization.

Recently the function of the calcium receptor has been shown to be positively modulated by certain L-amino acids (10, 11). This finding may be physiologically relevant as nutrient and Ca<sup>2+</sup> homeostasis may be coordinately regulated.

We recently reported that Zn<sup>2+</sup> has complex effects on the functional properties of the  $\beta_2$ AR (12). Zn<sup>2+</sup> binding to a high affinity site (IC<sub>50</sub> ~5  $\mu$ M) enhances agonist affinity, whereas Zn<sup>2+</sup> binding to one or more low affinity sites (IC<sub>50</sub> >500  $\mu$ M) inhibits antagonist binding and yet slows antagonist dissociation. To identify the Zn<sup>2+</sup> binding site responsible for the positive allosteric effect of Zn<sup>2+</sup> on agonist affinity, we mutated seven histidines located in intracellular and extracellular hydrophilic sequences connecting transmembrane (TM) domains. Mutation of His-269 dramatically reduced the effect of Zn<sup>2+</sup> on agonist affinity. Mutations of other histidines had no effect. Further mutagenesis of residues predicted to be adjacent to His-269 in the three-dimensional structure of the  $\beta_2$ AR revealed that Cys-265 and Glu-225 are also required to achieve the full allosteric effect of Zn<sup>2+</sup> on agonist binding. Our results suggest that bridging of the cytoplasmic extensions of TM5 and TM6 by Zn<sup>2+</sup> facilitates agonist binding. These results are in agreement with recent biophysical studies demonstrating that agonist binding leads to movement of TM6 relative to TM5.

### EXPERIMENTAL PROCEDURES

**Site-directed Mutagenesis**—The cDNA sequence encoding the human  $\beta_2$ AR, epitope-tagged at the amino terminus with a cleavable influenza-hemagglutinin signal sequence followed by the FLAG epitope (IBI, New Haven, CT) and tagged with six histidines at the carboxyl terminus, was used as a template for mutagenesis (13). This cDNA was subcloned in baculovirus expression vector pACMP2 at *Bam*HI/*Eco*RI site. Mutations were generated by the polymerase chain reaction-mediated mutagenesis using *Pfu* polymerase (Stratagene). The polymerase-generated DNA fragments were subcloned into pACMP2 plasmid, and the

\* This work was supported in part by National Institute of Health Grant 5-RO1-NS28471 and the Mathers Charitable Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Stanford University School of Medicine, 157 Beckman Center, 279 Campus Dr., Stanford, CA 94305. Tel.: 650-723-7069; Fax: 650-498-5092; E-mail: kobilka@cmgm.stanford.edu.

<sup>1</sup> The abbreviations used are: GPCR, G protein-coupled receptors;  $\beta_2$ AR,  $\beta_2$ -adrenergic receptor; WT, wild type; TM, transmembrane domain; DHA, dihydroalprenolol; ISO, (–)-isoproterenol; PD 81,723, (2-amino-4,5-dimethyl-3-thienyl)-[3-(trifluoromethyl)phenyl]methanone; GTP $\gamma$ S, guanosine 5'-O-(thiotriphosphate).

single mutants H93A, H172A, H178A, H241A, H256A, H269A, H296A, and E225A and double mutants E225A/H269A and E225A/C265A were confirmed by DNA sequencing and restriction analysis. The construct C265A $\beta_2$ AR was a gift from Dr. Charles Parnot.

**Mammalian Cell Culture**—HEK293 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Stable cell lines were obtained for wild-type  $\beta_2$ AR (WT $\beta_2$ AR) and the mutants in the presence of 0.5 mg/ml G418.

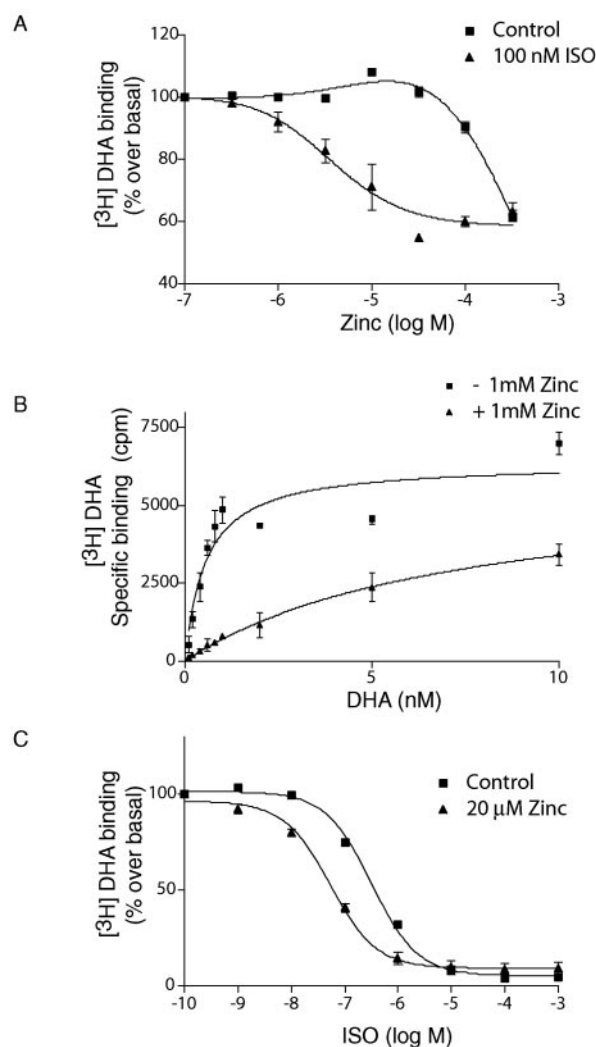
**Expression of the Receptors in SF9 Insect Cells**—SF-h $\beta_2$ -6H and the mutant constructs in the baculovirus expression vector pACMP2 were co-transfected with linearized Sapphire™ Baculovirus DNA into SF9 insect cells using the Insectin Plus transfection kit (Invitrogen). The resulting viruses were harvested 4–5 days and amplified for another 4–5 days to obtain a high titer virus. SF9 insect cells were grown in suspension culture in SF-900 II medium (Invitrogen) containing 5% fetal calf serum (Gemini, Calabasas, CA) and 0.1 mg/ml gentamicin (Invitrogen). For membrane preparation the cells were grown in 100-ml cultures. Cells were infected with a 1:100 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 for 10 min at 5000  $\times g$ . The resulting cell pellets were kept at  $-80^\circ\text{C}$  until further use.

**Membrane Preparation**—All of the membrane preparation steps were done at  $4^\circ\text{C}$  as described elsewhere (12). Cells were harvested and washed once with phosphate-buffer saline, recentrifuged, and then resuspended in lysis buffer (10 mM Tris-HCl, pH 7.5, with 1 mM EDTA) containing protease inhibitors (leupeptin and benzamide at a final concentration of 10  $\mu\text{g}/\text{ml}$ ) and lysed using 25 strokes of a Dounce homogenizer. Nuclei and unbroken cells were removed by centrifugation (5 min at 500g). The supernatant was removed and centrifuged at 40,000  $\times g$  for 30 min. The resulting pellet was resuspended in 20 ml of 10 mM Tris-HCl, pH 7.5, buffer alone containing protease inhibitors and recentrifuged. Membranes were resuspended at 0.5–1.5 mg/ml in binding buffer (75 mM Tris-HCl, pH 7.5) and stored at  $-80^\circ\text{C}$  until use.

**Binding Assays**—All of the binding assays were done on SF9 membranes expressing either the WT $\beta_2$ AR or mutant receptors. The saturation binding assays were done by incubating the membranes (20–50  $\mu\text{g}$ ) with 10 different concentrations of the antagonist [<sup>3</sup>H]DHA between 100 pM and 20 nM with or without 1 mM Zn(II). Competition assays were done by incubating the membranes with different concentrations of agonist isoproterenol ( $10^{-9}$  to  $10^{-3}$  M) with or without 20  $\mu\text{M}$  Zn(II). Zn<sup>2+</sup> modified competition assays were also done similar to competition assays expect that membranes were incubated with different concentrations of Zn(II) (0.3–300  $\mu\text{M}$ ) in the presence or absence of 100 nM isoproterenol. All of the assays were performed for 1 h at room temperature with shaking at 230 rpm. Competition assays were carried out with 1 nM [<sup>3</sup>H]DHA around the  $K_D$ . Binding data were analyzed by nonlinear regression analysis using Prism from GraphPad Software, San Diego, CA. Inhibitory constant ( $K_I$  values) were calculated from  $\text{IC}_{50}$  values using the Cheng-Prusoff equation:  $K_I = \text{IC}_{50}/(1 + [\text{ligand}]/K_D)$ .

**Dissociation Rate Kinetic Assay**—The effect of Zn<sup>2+</sup> on the rate of antagonist dissociation from  $\beta_2$ AR was examined by measuring the  $k_{\text{off}}$  in the absence and presence of 1 mM Zn<sup>2+</sup>. Membranes were suspended in 75 mM Tris-HCl, pH 7.5, with 1 nM [<sup>3</sup>H]DHA for 30 min at room temperature (shaking at 230 rpm). At time zero, total binding was determined, and a saturating amount of cold alprenolol (final  $10^{-5}$  M) or cold alprenolol (final  $10^{-5}$  M) and ZnCl<sub>2</sub> (final 1 mM) was added to tubes containing membranes and [<sup>3</sup>H]DHA. Bound [<sup>3</sup>H]DHA was measured at 5-min intervals.

**cAMP Accumulation**—The production of cAMP was determined by adenylyl cyclase activation FlashPlate assay (PerkinElmer Life Sciences), in which 96-well plates are coated with solid scintillant to which anti-cyclic cAMP antibody has been bound. Briefly, HEK293 cells expressing stable human  $\beta_2$ AR and mutants were detached, washed four times in 1 $\times$  phosphate-buffered saline without Ca<sup>2+</sup>/Mg<sup>2+</sup>, and then resuspended to a density of  $\sim 2 \times 10^6$  cells/ml in stimulation buffer (1 $\times$  phosphate-buffered saline without calcium/magnesium, with 700  $\mu\text{M}$  3-isobutyl-1-methylxanthine, 0.1% protease-free bovine serum albumin, and 0.09% chloroacetamide) from PerkinElmer Life Sciences. Ligands (25  $\mu\text{l}$  each) were diluted in Milli-Q water with various concentrations and dispensed to the FlashPlate. Resuspended whole cells (50  $\mu\text{l}$ ) were added to the ligand-loaded plate and stimulated at  $37^\circ\text{C}$  for 10 min before lysing cells with 100  $\mu\text{l}$  of Detection Buffer (Invitrogen) containing [<sup>125</sup>I]cAMP, permeabilizer, and 0.09% sodium azide as provided by the manufacturer. After 2 h of incubation at room temperature, radioactivity was counted. To determine the concentrations of cAMP in the sample, cAMP standards were run in the same plate and expressed as pmol/well. The expression level of mutant  $\beta_2$ ARs in stable



**FIG. 1. Effect of Zn<sup>2+</sup> on antagonist and agonist binding in WT $\beta_2$ AR expressed in SF9 membranes.** A, inhibition of antagonist binding by different concentrations of Zn<sup>2+</sup> in the presence or absence of 100 nM isoproterenol. The assays were performed as described under "Experimental Procedures." B, saturation binding of [<sup>3</sup>H]DHA (100 pM–10 nM) on membranes expressing WT $\beta_2$ AR receptor (17 pmol/mg protein) in the presence or absence of 1 mM Zn<sup>2+</sup>. Data were fit to a monophasic saturation hyperbolae. C, effect of Zn<sup>2+</sup> on agonist binding. Isoproterenol competition assays were performed with 1 nM [<sup>3</sup>H]DHA in the presence or absence of 20  $\mu\text{M}$  Zn<sup>2+</sup>. Data represent the mean of 2–4 experiments. Each experiment was done in triplicates.

HEK293 cell lines was assessed using a single saturating concentration of [<sup>3</sup>H]dihydroalprenolol (10 nM).

**Miscellaneous**—Protein concentration was determined using the Bio-Rad DC protein assay kit.

## RESULTS

**The Effect of Zn<sup>2+</sup> on Binding of Agonist and Antagonists to the  $\beta_2$ AR**—To examine the effect of Zn<sup>2+</sup> on both agonist and antagonist binding to the  $\beta_2$ AR, we performed a modified competition experiment in which the  $\beta_2$ AR is incubated with 1 nM [<sup>3</sup>H]DHA and varying concentrations of Zn<sup>2+</sup> in the presence or absence of 100 nM isoproterenol. These studies are also done with 10  $\mu\text{M}$  GTP $\gamma$ S to eliminate the effects of G<sub>s</sub> on agonist binding. In the absence of isoproterenol we observed a reduction of [<sup>3</sup>H]DHA binding only at relatively high concentrations of Zn<sup>2+</sup> (>500  $\mu\text{M}$ ). Agonist binding is more sensitive to Zn<sup>2+</sup>. In the presence of increasing concentrations of Zn<sup>2+</sup>, 100 nM isoproterenol becomes more effective at displacing [<sup>3</sup>H]DHA (Fig. 1A). The maximal effect of Zn<sup>2+</sup> on isoproterenol affinity occurs at  $\sim 20$   $\mu\text{M}$  with an  $\text{IC}_{50}$  of 3.0  $\mu\text{M}$  (12). These effects of

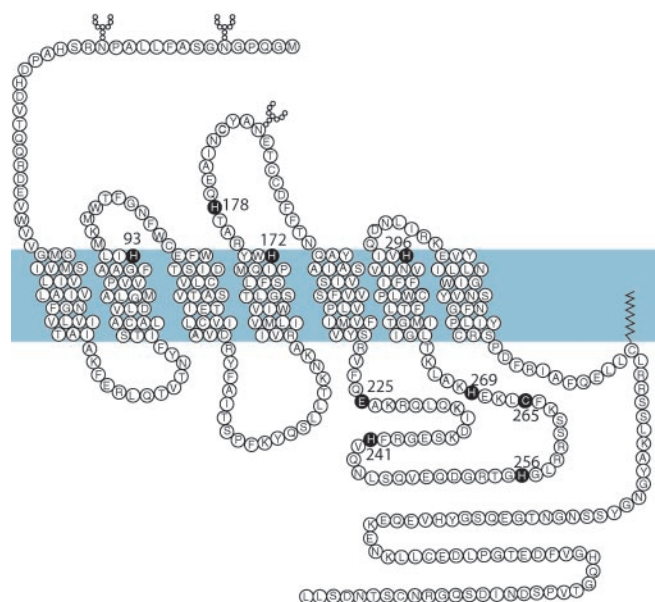


FIG. 2. Two-dimensional representation of human  $\beta_2$ AR. The highlighted residues have been mutated to identify putative  $Zn^{2+}$  binding sites.

$Zn^{2+}$  can also be observed in more conventional saturation (Fig. 1B) and competition (Fig. 1C) binding studies. At 1 mM  $Zn^{2+}$  we observe a decrease in  $B_{max}$  and an increase in the  $K_D$  for [ $^3$ H]DHA (Fig. 1B). In contrast, the affinity of the  $\beta_2$ AR for isoproterenol is enhanced in the presence of 20  $\mu$ M  $Zn^{2+}$  (Fig. 1C).

**Identification of the Allosteric Binding Site for  $Zn^{2+}$** —Histidines are often involved in the formation of  $Zn^{2+}$  binding sites in proteins (14, 15). We hypothesized that the  $Zn^{2+}$  binding site responsible for the positive allosteric effect on agonist binding to the  $\beta_2$ AR would be located in extracellular loops. However, mutations of histidines 93, 172, 178, and 296 (Fig. 2) had no effect on the positive allosteric effect of  $Zn^{2+}$  (data not shown). We therefore examined the role of cytoplasmic histidines (His-241, His-256, and His-269) (Fig. 2). Of these, only H269A exhibited a reduced response to  $Zn^{2+}$ . The effect of  $Zn^{2+}$  on both agonist and antagonist affinity is nearly abolished in H269A (Fig. 3; Tables I and II). Of interest, we found that H269A mutation does not alter the effect of 1 mM  $Zn^{2+}$  on the dissociation rate of [ $^3$ H]DHA (data not shown), indicating that the site responsible for the positive allosteric effect of  $Zn^{2+}$  on agonist binding is different from the binding site responsible for the effect of  $Zn^{2+}$  on the dissociate rate of antagonists.

High affinity  $Zn^{2+}$  binding requires coordination by at least two amino acids. In addition to histidine, cysteine, aspartate, and glutamate can contribute to the formation of a  $Zn^{2+}$  binding site (16, 17). We therefore used a three-dimensional model based on rhodopsin (18) to identify cysteines as well as acidic residues in proximity to His-269. This analysis identified the closest candidates as Cys-265 on the cytoplasmic end of TM6 and Glu-225 on the cytoplasmic end of TM5. Mutation of Glu-225 and Cys-265 to alanine had no significant effect on either agonist or antagonist affinity in the absence of  $Zn^{2+}$  (Tables I and II). However, both mutant receptors had altered agonist and antagonist responses to  $Zn^{2+}$ . For both mutants there was a smaller decrease in antagonist affinity in the presence of 1 mM  $Zn^{2+}$  (Table I) and a smaller increase in agonist affinity in the presence of 20  $\mu$ M  $Zn^{2+}$  (Table II). The effect of  $Zn^{2+}$  on agonist affinity is almost abolished for the double mutant E225A/H269A and completely abolished when C265A is combined with H269A (Tables I and II, and Fig. 4).

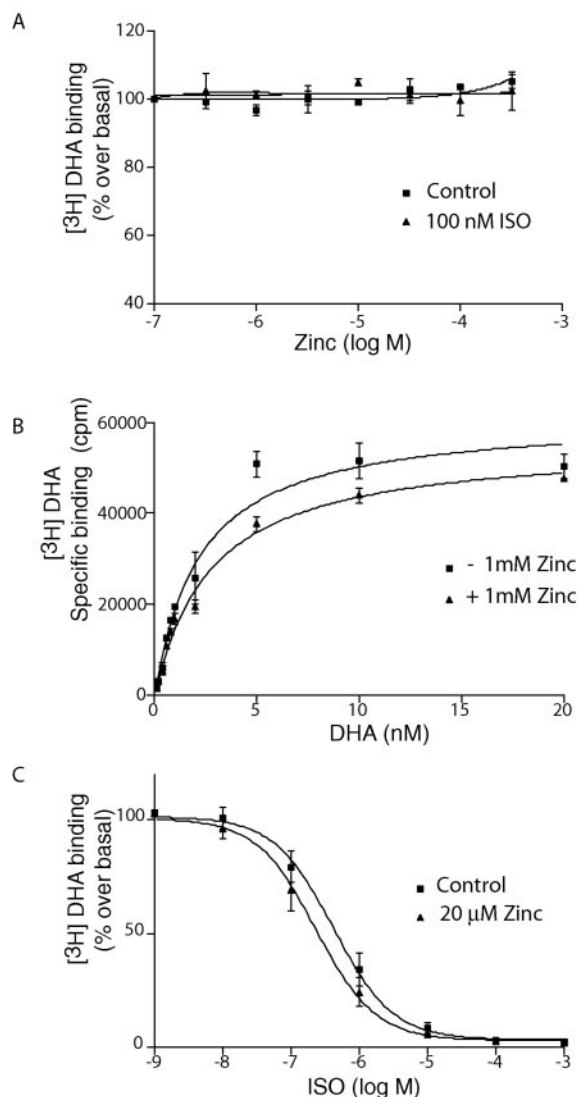


FIG. 3. Effect of  $Zn^{2+}$  on antagonist and agonist binding in H269A $\beta_2$ AR expressed in SF9 membranes. A, inhibition of antagonist binding by different concentrations of  $Zn^{2+}$  in the presence or absence of 100 nM isoproterenol. The assays were performed as described under "Experimental Procedures." B, saturation binding of [ $^3$ H]DHA (100 pM–10 nM) on membranes expressing H269A $\beta_2$ AR receptor (4.4 pmol/mg protein) in the presence or absence of 1 mM  $Zn^{2+}$ . Data were fitted to monophasic saturation hyperbolae. C, effect of  $Zn^{2+}$  on agonist binding. Isoproterenol competition assays were performed with 1 nM [ $^3$ H]DHA in the presence or absence of 20  $\mu$ M  $Zn^{2+}$ . Data represent the mean of 2–4 experiments, each done in triplicate.

**$Zn^{2+}$  Augmentation of ISO-stimulated cAMP Accumulation**—We previously reported that low concentrations of  $Zn^{2+}$  enhance cAMP accumulation by submaximal concentrations of isoproterenol in intact cells (12). To determine whether the  $Zn^{2+}$  site formed by His-269, Cys-265, and Glu-225 is also responsible for the effect of  $Zn^{2+}$  on cAMP accumulation, we generated stable cell lines expressing the wild-type  $\beta_2$ AR as well as H269A, C265A, C265A/H269A, and E225A/H269A. Fig. 5A shows the effect of  $Zn^{2+}$  on cAMP accumulation in the presence and absence of 0.1 nM isoproterenol in nontransfected HEK293 cells and in HEK293 cells stably expressing the wild-type  $\beta_2$ AR. Isoproterenol at 0.1 nM stimulates a submaximal cAMP response that is augmented by 10  $\mu$ M  $Zn^{2+}$ . As shown in Fig. 5B, this effect of  $Zn^{2+}$  was also observed in all of the cell lines expressing mutant receptors. Therefore, the  $Zn^{2+}$  binding site responsible for allosteric modulation of agonist binding is

TABLE I  
 Binding properties of WT and  $\beta_2$ AR mutants

Saturation experiments using [ $^3$ H]DHA were performed on membranes expressing  $\beta_2$ AR or the mutants from SF9 membranes. Values represent the means of two or more experiments  $\pm$  S.E. Each experiment was done in triplicate. All calculations were obtained using the Graphpad software program.

Mutants	$K_D$		$K_D$ ratio	% Change in $B_{max}$
	-1 mM $Zn^{2+}$	+1 mM $Zn^{2+}$		
	<i>nM</i>			
WT	0.54 $\pm$ 0.14	7.4 $\pm$ 2.1	13.7	51
H269A	1.182 $\pm$ 0.16	2.0 $\pm$ 0.41	1.69	24
C265A	0.44 $\pm$ 0.06	0.84 $\pm$ 0.19	1.9	52
E225A	0.91 $\pm$ 0.11	2.2 $\pm$ 0.32	2.4	27.6
E225A/H269A	0.43 $\pm$ 0.10	1.7 $\pm$ 0.36	3.95	16.8
C265A/H269A	0.41 $\pm$ 0.05	1.76 $\pm$ 0.15	4.29	31.5

 TABLE II  
 Pharmacological characterization of WT and mutant  $\beta_2$ AR receptors

Agonist binding properties of WT and mutants in the presence or absence of 20  $\mu$ M zinc are reported here. The competition assays were performed as described under "Experimental Procedures" in the presence of 10  $\mu$ M GTP $\gamma$ S. The results are reported as  $K_I$  (nM) and the  $K_I$  ratio (+zinc)/(-zinc) is given. Data were obtained from 2-5 experiments performed in triplicate.

Mutants	$K_I$		$K_I$ ratio
	-Zinc	+Zinc	
	<i>nM</i>		
WT	110	18.5	0.168
H269A	220	120	0.54
C265A	60	40	0.66
E225A	91	35	0.38
E225A/H269A	31.5	25.6	0.81
C265A/H269A	22	25	1.13

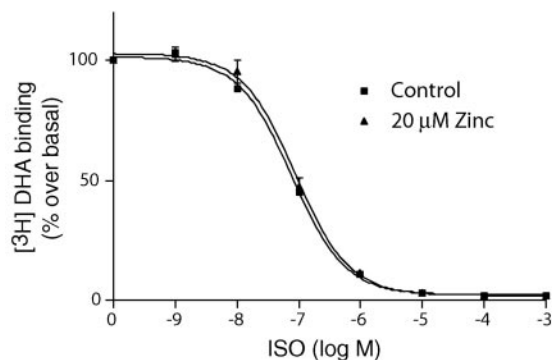


FIG. 4. Effect of  $Zn^{2+}$  on agonist affinity for C265A/H269A $\beta_2$ AR mutant expressed in SF9 membranes. Isoproterenol competition assays were performed with 1 nM [ $^3$ H]DHA in the presence or absence of 20  $\mu$ M  $Zn^{2+}$ . Data represent the mean  $\pm$  S.D. of three independent experiments performed in triplicate.

not responsible for the effect of  $Zn^{2+}$  on cAMP accumulation in intact cells.

#### DISCUSSION

Allosteric ligands have been identified for only a small number of GPCRs including the M2 muscarinic receptor (19), the A1 adenosine receptor (3), and the Ca $^{2+}$ -sensitive receptor (11). Binding of the well characterized muscarinic allosteric ligand gallamine has been mapped to extracellular loops 2 and 3 (20, 21). It has been proposed that, when bound to gallamine, extracellular loops 2 and 3 may form a plug over the binding pocket, thereby slowing the dissociation rate (22).

Another well characterized allosteric modulator of GPCR function is ionized sodium. Sodium ions act as negative allosteric regulators for a number of Gi-coupled receptors including the  $\alpha_{2A}$  adrenergic receptor and the D2 and D4 dopamine receptor. In contrast to the extracellular location of the allo-

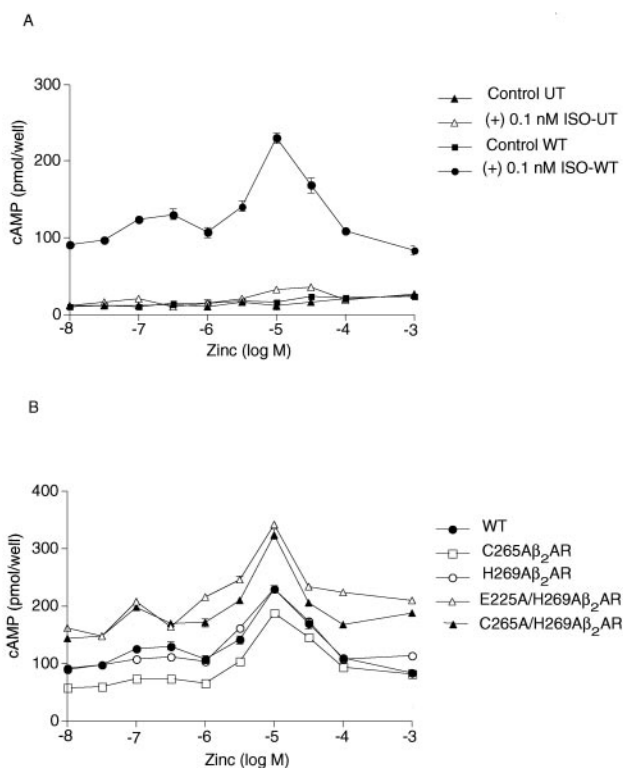


FIG. 5.  $Zn^{2+}$  augmentation of cAMP accumulation stimulated by a submaximal concentration of ISO. A, effect of different concentrations of  $Zn^{2+}$  on cAMP accumulation in the presence and absence of 0.1 nM ISO in untransfected cells (UT) and cells expressing WT $\beta_2$ AR. B, effect of different concentrations of  $Zn^{2+}$  on cAMP accumulation in the presence of 0.1 nM ISO in cells stably expressing WT $\beta_2$ AR and H269A, C265A, C265A/H269A, and E225A/H269A. The expression of stable WT $\beta_2$ AR and mutants are as follows (in pmol/mg protein): WT $\beta_2$ AR, 14.9; H269A, 16.6; C265A, 19.7; C265A/H269A, 21.1; E225A/H269A, 26.7. Data represent the mean of three independent experiments performed in triplicate.

steric binding site for gallamine in muscarinic receptors, sodium sensitivity is mediated at the cytoplasmic surface of the receptor. A highly conserved Asp at the cytoplasmic end of TM2 has been shown to be critical for this effect of sodium (6, 9).

Our mutagenesis studies localize the binding site responsible for the positive allosteric effect of  $Zn^{2+}$  on agonist affinity to the third intracellular loop. This  $Zn^{2+}$  binding site consists of Cys-265 and His-269 on the cytoplasmic extension of TM6 and Glu-225 on the cytoplasmic extension of TM5. This location is of particular interest because recent biophysical studies from our laboratory demonstrate that agonist binding is associated with a movement of Cys-265 on TM6 relative to TM5 (23). These studies were performed on purified  $\beta_2$ AR labeled at Cys-265 with fluorescein. Moreover, cysteine cross-linking studies on the M3 muscarinic receptor provide evidence for agonist-in-

duced movement of the cytoplasmic end of TM5 relative to TM6 (24). Thus,  $Zn^{2+}$  may form a bridge between TM5 and TM6. This bridge is likely to alter the position of TM5 relative to TM6. On the basis of these observations, we speculate that  $Zn^{2+}$  binding approximates or stabilizes the conformational changes induced by agonists. It is of interest that this  $Zn^{2+}$  binding site is not responsible for the ability of  $Zn^{2+}$  to augment cAMP accumulation by submaximal concentrations of isoproterenol (Fig. 5) or for the effect of  $Zn^{2+}$  on antagonist dissociation (data not shown).

In conclusion, we have identified the  $Zn^{2+}$  binding site responsible for the positive effect of  $Zn^{2+}$  ions on agonist binding to the  $\beta_2$ AR. Our results suggest that  $Zn^{2+}$  stabilizes an orientation of TM6 relative to TM5 that favors agonist binding and inhibits antagonist binding. These results provide further evidence for the role of TM5 and TM6 in agonist-induced conformational changes.

## REFERENCES

1. Tucek, S., and Proska, J. (1995) *Trends Pharmacol. Sci.* **16**, 205–212
2. Jakubik, J., Bacakova, L., El-Fakahany, E. E., and Tucek, S. (1997) *Mol. Pharmacol.* **52**, 172–179
3. Bhattacharya, S., and Linden, J. (1995) *Biochim. Biophys. Acta* **1265**, 15–21
4. Musser, B., Mudumbi, R. V., Liu, J., Olson, R. D., and Vestal, R. E. (1999) *J. Pharmacol. Exp. Ther.* **288**, 446–454
5. Limbird, L. E. (1984) *Am. J. Physiol.* E59–E68
6. Horstman, D. A., Brandon, S., Wilson, A. L., Guyer, C. A., Cragoe, E. J., and Limbird, L. E. (1990) *J. Biol. Chem.* **265**, 21590–21595
7. Motulsky, H. J., and Insel, P. A. (1983) *J. Biol. Chem.* **258**, 3913–3919
8. Neve, K. A. (1991) *Mol. Pharmacol.* **39**, 570–578
9. Schetz, J. A., and Sibley, D. R. (2001) *J. Pharmacol. Exp. Ther.* **296**, 359–363
10. Conigrave, A. D., Quinn, S. J., and Brown, E. M. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97**, 4814–4819
11. Conigrave, A. D., Quinn, S. J., and Brown, E. M. (2000) *Trends Pharmacol. Sci.* **21**, 401–407
12. Swaminath, G., Steenhuis, J., Kobilka, B., and Lee, T. W. (2002) *Mol. Pharmacol.* **61**, 65–72
13. Kobilka, B. K. (1995) *Anal. Biochem.* **231**, 269–271
14. Harvey, R. J., Thomas, P., James, C. H., Wilderspin, A., and Smart, T. G. (1999) *J. Physiol.* **520**, 53–64
15. Regan, L. (1995) *Trends Biochem. Sci.* **20**, 280–285
16. Vallee, B. L., and Falchuk, K. H. (1993) *Physiol. Rev.* **73**, 79–118
17. Maret, W. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98**, 12325–12327
18. 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
19. Ellis, J., and Seidenberg, M. (2000) *Mol. Pharmacol.* **58**, 1451–1460
20. Gnagey, A. L., Seidenberg, M., and Ellis, J. (1999) *Mol. Pharmacol.* **56**, 1245–1253
21. Leppik, R. A., Miller, R. C., Eck, M., and Paquet, J. L. (1994) *Mol. Pharmacol.* **45**, 983–990
22. Shi, L., and Javitch, J. A. (2002) *Annu. Rev. Pharmacol. Toxicol.* **42**, 437–467
23. Ghanouni, P., Steenhuis, J. J., Farrens, D. L., and Kobilka, B. K. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98**, 5997–6002
24. Ward, S. D., Hamdan, F. F., Bloodworth, L. M., and Wess, J. (2002) *J. Biol. Chem.* **277**, 2247–2257