

Determinants of Ligand Subtype-Selectivity at α_{1A} -Adrenoceptor Revealed Using Saturation Transfer Difference (STD) NMR

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Supporting Information



ABSTRACT: α_{1A} and α_{1B} -adrenoceptors (α_{1A} -AR and α_{1B} -AR) are closely related G protein-coupled receptors (GPCRs) that modulate the cardiovascular and nervous systems in response to binding epinephrine and norepinephrine. The GPCR gene superfamily is made up of numerous subfamilies that, like α_{1A} -AR and α_{1B} -AR, are activated by the same endogenous agonists but may modulate different physiological processes. A major challenge in GPCR research and drug discovery is determining how compounds interact with receptors at the molecular level, especially to assist in the optimization of drug leads. Nuclear magnetic resonance spectroscopy (NMR) can provide great insight into ligand-binding epitopes, modes, and kinetics. Ideally, ligand-based NMR methods require purified, well-behaved protein samples. The instability of GPCRs upon purification in detergents, however, makes the application of NMR to study ligand binding challenging. Here, stabilized α_{1A} -AR and α_{1B} -AR variants were engineered using Cellular High-throughput Encapsulation, Solubilization, and Screening (CHESS), allowing the analysis of ligand binding with Saturation Transfer Difference NMR (STD NMR). STD NMR was used to map the binding epitopes of epinephrine and A-61603 to both receptors, revealing the molecular determinants for the selectivity of A-61603 for α_{1A} -AR over α_{1B} -AR. The use of stabilized GPCRs for ligand-observed NMR experiments will lead to a deeper understanding of binding processes and assist structure-based drug design.

E nvironmental sensing and cell–cell communication are vital processes in all multicellular organisms. G Protein-Coupled Receptors (GPCRs) are heptahelical membrane proteins that are the major means by which cells sense and respond to extracellular stimuli.¹ A typical GPCR contains a binding site for stimulating ligands (agonists) accessible from the extracellular side of the heptahelical domains, whereas the cytoplasmic side houses binding sites for effector proteins such as G proteins and β -arrestins.² Members of the GPCR family have evolved to sense a diverse array of stimuli, reflecting the intrinsic adaptability of the GPCR scaffold. The fundamental role that these receptors play in most physiological pathways makes GPCRs the major class of drug targets.^{3,4} Conversely, only approximately 15% of known GPCRs are currently

targeted by prescription drugs, often despite extensive research linking them to disease states.^{3,4} A major reason for this discrepancy is that most GPCRs are grouped into subfamilies consisting of several closely related receptor subtypes, which all respond to the same endogenous agonists but may modulate different physiological processes. In therapy, however, it will often be necessary to selectively activate or inhibit only one of the subtypes. Target validation and selective drug targeting of specific subtypes is challenging because highly similar binding

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sites of such homologues make the development of selective ligands difficult, and less selective ligands can lead to off-target side-effects.

 α_{1A} - and α_{1B} -adrenoceptors (α_{1A} -AR and α_{1B} -AR) modulate the cardiovascular and nervous systems and are an example of two closely related GPCR subtypes that are activated by the same endogenous agonists, epinephrine and norepinephrine. α_{1A} -AR and α_{1B} -AR are clinically targeted by nonsubtype selective α_1 -AR blockers for the treatment of hypertension and Raynaud's disease, a condition where arterial spasms cause episodes of reduced blood flow.⁵ Both receptors are highly expressed in the myocardium and the central nervous system (CNS), and by using transgenic mouse models these receptors have been shown to mediate opposing responses to epinephrine and norepinephrine release.⁵ This makes their selective targeting of great importance for cardioprotection during heart failure, epileptic seizures, and neuroprotection in neurodegenerative diseases.⁵ Unfortunately, the elucidation and clinical targeting of the individual roles of α_{1A} -AR and α_{1B} -AR have remained difficult due to the lack of subtype-selective agonists that are efficacious in vivo.

With the recent advances in GPCR structural biology, it is hoped that a deeper understanding of subtle structural differences between GPCR subtypes will enable the structurebased drug design (SBDD) of more selective tool compounds and drugs. To date, however, there are no crystal structures of an α_1 -AR. This reflects the fact that routine examination of GPCR structure and function with biophysical approaches is not trivial, a problem exacerbated by the dynamic nature of GPCRs and their instability upon purification.^{6,7} For purification, GPCRs must be solubilized from cell membranes using detergents, a step that has hindered GPCR structural biology due to the instability of these proteins in detergent micelles.⁷ Ligand-GPCR binding and subsequent receptor activation are dynamic processes, for which the application of NMR methods can provide insight to augment the static structural knowledge provided by X-ray crystallographic studies.

Whereas technical advances in recent years have resulted in the crystal structure determination of dozens of different GPCRs,^{6,8} solution-state NMR studies have been mostly on the β_2 -AR,^{9–18} with several other studies on thermostabilized Turkey β_1 -AR,^{19,20} A_{2A}-adenosine,^{21–23} μ -opioid receptor,^{24,25} κ -opioid receptor,²⁶ and chemokine receptor CXCR4.^{27,28} These studies include protein-observed NMR, where spectra of isotopically labeled GPCR samples are acquired in the presence of various ligands, and ligand-observed NMR, where changes in the NMR spectra of ligands are monitored in the presence of an unobservable receptor.

Saturation transfer difference (STD) NMR is an example of a ligand-observed method that is widely used for characterizing ligand-protein interactions, compound screening, ligand affinity determination, and epitope mapping with traditionally soluble proteins.²⁹ STD NMR consists of applying several seconds of semiselective irradiation that only excites protein protons. This excitation is then transferred, by Nuclear Overhauser Effect (NOE) and spin diffusion mechanisms, to ligand protons that are in close contact with the protein. Comparison with a reference spectrum, where protein protons are not irradiated, reveals which ligand protons make intimate contacts with the protein. With STD NMR, only the ligand protons are monitored, meaning no labeling is required and the protein can be maintained at a relatively low concentration (~1 to $10 \ \mu$ M).²⁹ Magnetization transfer between protein and

ligand is highly dependent on intermolecular atomic distances. Thus, the relative intensities of proton STD signals throughout the ligand can be used to map ligand epitopes. Ideally, epitope mapping with STD NMR requires pure, solubilized protein, multiple acquisition cycles (>6 h acquisition per sample), and temperatures that would denature most detergent-solubilized GPCRs.

To date STD NMR has been applied to detect the binding of the sweet tasting protein Brazzein to the large extracellular domains of the sweet receptor in membrane preps³⁰ and cholesterol to detergent solubilized β_2 -AR.¹⁵ However, STD NMR has not been applied to detect binding of ligands to the classical, orthosteric ligand binding site located within the transmembrane domains. Similarly, no epitope mapping studies have been performed using STD NMR on GPCR-binding ligands. The mapping of GPCR-binding ligand epitopes, especially for weakly binding agonists, with STD NMR would be an advancement for the field because solving crystal structures of weakly binding agonist-GPCR complexes is challenging. Of the hundreds of structures solved to date, there are only two structures of GPCRs bound to agonists with micromolar affinities, the adrenaline bound β_2 -AR,³¹ which required an active-state stabilizing nanobody and the dobutamine bound turkey β_1 -AR structure,³² which used a thermostabilized GPCR. Here, we describe the evolution of stabilized variants of human α_{1A} -AR and α_{1B} -AR using Cellular Highthroughput Encapsulation, Solubilization, and Screening (CHESS).33 These receptors exhibited the stability required for STD NMR, enabling the analysis and epitope mapping of epinephrine, and the α_{1A} -AR-selective agonist A-61603, to α_{1A} -AR and α_{1B} -AR. Thus, the use of STD NMR represents an attractive means to identify differences in ligand binding epitopes at closely related receptor subtypes, information that can be used to model receptor binding sites and guide SBDD.

RESULTS AND DISCUSSION

Stabilization of α_{1A} -AR and α_{1B} -AR Using CHESS. CHESS is a directed evolution method that can evolve populations of GPCR mutants for improved stability in detergents.³³ The technique works by encapsulating E. coli cells, where each cell expresses a unique GPCR mutant library member, into detergent-resistant microcapsules. GPCR proteins embedded in the cell membranes can then be detergentsolubilized within the microcapsules and probed for stability using receptor-specific fluorescent ligands. The variants that are detergent-stable can bind fluorescent ligands, thus conferring high fluorescence to the microcapsules they are contained within, allowing fluorescence-activated cell sorting (FACS) of improved mutants. Because the plasmids encoding each receptor mutant are retained within the CHESS microcapsules, the genetic information corresponding to detergent-stable receptors can be recovered and the process repeated in an evolutionary manner until the desired receptor stability is achieved.

A total of three rounds of error-prone PCR and 11 rounds of CHESS were required to reach the desired level of stability for α_{1A} -AR (Supporting Information Figure 1), defined as the CHESS population exhibiting high levels of specific BODIPY-FL-prazosin (QAPB [Quinazoline Piperazine Bodipy]) binding after incubation with *n*-decyl- β -D-maltopyranoside (DM) for several hours. Conversely, only two rounds of error-prone PCR and six rounds of CHESS were required to evolve the α_{1B} -AR to a similar level of stability (Supporting Information Figure 2).



Figure 1. Characterization of stabilized α_1 -ARs. Temperature denaturation curves of (A) purified α_{1A} -AR mutants and (B) α_{1B} -AR mutants heated in the absence (Apo, dashed curves) or presence of QAPB (Holo, solid curves) for 30 min in 0.1% DDM. (C) QAPB competition binding for 2 h at 4 °C against purified α_{1A} -AR A4 (solid circles and lines) and α_{1B} -AR #15 (open squares and dashed lines) with prazosin (Praz, black), epinephrine (Epi, blue), and phenylephrine (Phe, red). (D) Competition binding of ³H-prazosin to COS-7 cells overexpressing: WT α_{1A} -AR (solid symbols and lines) or α_{1A} -AR A4 (open symbols and dashed lines) or (E) WT α_{1B} -AR (solid symbols and lines) and α_{1B} -AR #15 (open symbols and dashed lines) with phenylephrine (Phe, circles) or epinephrine (Epi, squares). (F) Measurement of agonist induced accumulation of IP₁ in COS-7 cells transfected with the angiotensin II (Ang II) receptor, AT₁, were included as an additional positive control.

Single clones were isolated from these populations, expressed, purified, and screened for detergent stability. After solubilization in DM for 3 h in the presence of 100 nM QAPB, 25 out of 32 α_{1A} -AR clones (Supporting Information Figure 3A) and 13 out of 15 α_{1B} -AR clones (Supporting Information Figure 3B) displayed significantly greater QAPB binding than solubilized WT receptors. Seven unique α_{1A} -AR mutants were identified that collectively contained 26 different amino acid substitutions (Supporting Information Figure 3C). Ten unique α_{1B} -AR mutants were identified that contained a total of 38 different mutations (Supporting Information Figure 3D). To identify the most stable mutants, each was tested for stability in DM over periods of several hours without a bound ligand. All the single clones displayed improved QAPB binding over WT (Supporting Information Figure 4A,B). Three α_{1A} -AR mutants (α_{1A} -AR-A4, α_{1A} -AR-F3, and α_{1A} -AR-H4) and three α_{1B} -AR mutants (α_{1B} -AR#4, α_{1B} -AR#12, and α_{1B} -AR#15) with the highest ligand binding after incubation for 5 h in DM were chosen for temperature stability (thermostability) assays in *n*-dodecyl- β -D-

maltopyranoside (DDM). Each of these variants displayed high stability when heated in the absence or presence of a bound ligand (Figure 1A and B). Comparison of the calculated $T_{\rm m}$ values determined that $\alpha_{\rm 1A}$ -AR A4 and $\alpha_{\rm 1B}$ -AR #15 were the most stable variants of each subtype when heated in the apostate, or with ligand bound (Table 1).

Characterization of Stabilized α_1 -**AR Variants.** α_{1A} -**AR** A4 contained 15 amino acid substitutions over WT α_{1A} -**AR**, whereas α_{1B} -**AR** #15 harbored nine mutations compared to WT α_{1B} -**AR**. The locations of these mutations were mapped onto homology models of both receptors (Supporting Information Figures 5 and 6). To allow simple reference of identical amino acid positions between each receptor, GPCRdb (GPCRdb.org) residue numbering³⁴ is used throughout. α_{1A} -**AR** A4 contains two mutations in the N-terminal extracellular domain (C14S and T15I), one mutation in TM1 (L36I or GPCRdb position 1.42x42), four mutations in TM2 (I65V, N67Y, L80M, and F86Y, at positions 2.43x43, 2.45x45, 2.58x57, and 2.64x63, respectively), one mutation in TM3 (G127A, at position

Table 1. Thermostability of Evolved α_1 -ARs Purified in DDM^{*a*}

receptor	$T_{\rm m}$ APO (°C)	$T_{\rm m}$ HOLO (°C)
WT α_{1A} -AR	N.A.	N.A.
α_{1A} -AR A4	40.9 ± 0.5	50.4 ± 0.3
α_{1A} -AR F3	$32.6 \pm 0.4^*$	$42.7 \pm 0.2^*$
α_{1A} -AR H4	$30.9 \pm 0.8^*$	49.4 ± 0.2
α_{1A} -AR A4 F312L	$28.3 \pm 0.6^{*}$	$43.3 \pm 0.5^*$
WT α_{1B} -AR	N.A.	N.A.
α_{1B} -AR #4	29.8 ± 0.4	$42.9 \pm 0.1^{\ddagger}$
α_{1B} -AR #12	32.9 ± 0.3	43.5 ± 0.3
α_{1B} -AR #15	32.7 ± 0.4	44.9 ± 0.7
α_{1B} -AR #15 F334L	$25.5 \pm 1.6^{\ddagger}$	$33.1 \pm 0.2^{\ddagger}$

^{*a*}Receptors were heated for 30 min at various temperatures in the absence (APO) or presence (HOLO) of QAPB. The WT receptors were unstable in DM, and thus no stability curves could be measured (N.A.). Data are the mean \pm SEM of three independent experiments. The * and ‡ indicate a statistical difference from α_{1A} -AR A4 and α_{1B} -AR #15, respectively, within each column, based on one-way ANOVA with Tukey multiple comparisons tests.

3.53x53) and TM4 (W151F, at position 4.50x50), one mutation in ECL2 (E171V), three mutations in TM7 (F312L, N322K, and P327L, at positions 7.39x38, 7.49x49, and 7.54x54), and one mutation between TM7 and helix 8 (S329Y). The F86Y and F312L mutations lie within the predicted epinephrine binding site, whereas L80M is near the binding site. The N322K mutation is in the NPxxY motif, which is important for receptor activation.

 α_{1B} -AR #15 contains one mutation in the N-terminus (G27D), one mutation in TM2 (L105Q, at position 2.64x63), one mutation in ICL2 (S150Y), two mutations in ECL2 (D191Y and E194G), one mutation in TM6 (T295M, at position 6.36x36), and one mutation in TM7 (F334L, at position 7.39x38). F334L is located at the top of the

Table 2.	Pharmacological	Characterization	of Evolved	α_1 -AR ^{<i>a</i>}

epinephrine binding site and is analogous to the F312L mutation found in α_{1A} -AR A4. The E194G mutation is in the extracellular vestibule, along the binding pathway for epinephrine into the predicted orthosteric binding site. Given the locations of these mutations, the binding of several prototypical α_1 -AR ligands to the stabilized receptors was measured.

Saturation binding assays revealed QAPB bound to the solubilized receptors with slightly weaker affinities than the $K_{\rm d}$ of around 2 nM that has been reported for this ligand on WT α_{1A} -AR and α_{1B} -AR expressing mammalian cells³⁵ (Supporting Information Figure 7A and Table 2). Competition binding determined that the endogenous agonist epinephrine, and the α_1 -AR-selective agonist phenylephrine, could bind both stabilized receptors solubilized in detergent, albeit with weaker affinities than expected (Figure 1C and Table 2). To enable direct comparison of ligand binding to the stabilized variants with their WT counterparts, whole-cell radioligand (³Hprazosin) binding assays were conducted using COS-7 cells overexpressing each receptor. While the affinity of ³H-prazosin for α_{1A} -AR WT and α_{1A} -AR A4 was not statistically different, all other ligands bound with weaker affinities to the stabilized receptors compared to the WT receptors (Figure 1D,E; Supporting Information Figure 7B,C and Table 2).

It is generally accepted that the engineering of stabilized GPCRs in a state bound to antagonists or inverse agonists can result in receptor mutants that prefer inactive states,³⁶ thus resulting in weaker agonist affinities. The ability of the stabilized receptors to signal in response to agonist binding was also measured. α_1 -ARs predominately couple to $G\alpha_{q/11}$ to activate phospholipase C- β (PLC β) and catalyze the formation of the second messenger, inositol 1,4,5-trisphosphate (IP₃), which is metabolized to inositol monophosphate (IP₁). Stimulation of COS-7 cells overexpressing α_{1A} -AR A4 and α_{1B} -AR #15 with epinephrine failed to increase cellular levels of

		α_{1A} -AR	α_{1A} -AR	α_{1B} -AR	α_{1B} -AR	α_{1A} -AR	α_{1B} -AR
	exp. type	WT	A4	WT	#15	A4 (A189S)	#15 (S208A)
QAPB $K_{\rm d}$ (nM)	sol. sat.	N.A.	11.6 ± 2.0	N.A.	8.5 ± 1.1	$16.3 \pm 2.4^*$	$8.0 \pm 0.9^{*}$
Praz pK _i	sol. comp.	N.A.	$8.0 \pm 0.05^{*}$	N.A	$7.9 \pm 0.04^{\ddagger}$	$6.9 \pm 0.15^{*,\ddagger,\dagger}$	$8.0\pm0.09^\dagger$
$\sim K_i$ (nM)			10		12.6	125.9	10
Epi pK _i	sol. comp.	N.A.	<3	N.A.	<3	N.D.	N.D.
$\sim K_i (\mu M)$			>1000		>1000		
Phe pK _i	sol. comp.	N.A.	$3.2 \pm 0.01^{*,\ddagger}$	N.A.	$2.7 \pm 0.05^{*,\dagger}$	$3.3 \pm 0.05^{\dagger}$	$2.7 \pm 0.14^{\ddagger}$
$\sim K_i (\mu M)$			631		2000	500	2000
A-61 pK_i	sol. comp.	N.A.	$4.4 \pm 0.03^{*^{\ddagger \dagger}}$	N.A.	$3.6 \pm 0.11^{*,\bullet}$	$5.0 \pm 0.15^{\ddagger, \bullet, \perp}$	$3.5 \pm 0.05^{\dagger,\perp}$
$\sim K_i (\mu M)$			40		250	10	316
³ H-Praz K _d (nM)	cells sat.	1.3 ± 0.3	7.3 ± 1.6	$0.7 \pm 0.3^{*}$	$20.9 \pm 6.7^*$	N.D.	N.D.
³ H-Praz Bmax	cells sat.	5400 ± 1300	23000 ± 7000	2900 ± 600	32000 ± 10000	N.D.	N.D.
Epi pK _i	cells comp.	$4.18 \pm 0.22^{*}$	<3*	$3.96 \pm 0.08^{\ddagger}$	<3 [‡]	N.D.	N.D.
$\sim K_i (\mu M)$		66	>1000	110	>1000		
Phe pK _i	cells comp.	$4.20 \pm 0.06^{*}$	$3.00 \pm 0.04^*$	$3.89 \pm 0.07^{\ddagger}$	$2.94 \pm 0.03^{\ddagger}$	N.D.	N.D.
$\sim K_i (\mu M)$		63	1000	129	1150		
Phe pEC ₅₀	cells CRE	$6.74 \pm 0.05^*$		$5.83 \pm 0.04^{\ddagger}$		N.D.	N.D.
~EC ₅₀ (µM)		0.18	-	1.48	-		

"Receptors were assayed for QAPB, prazosin (Praz), epinephrine (Epi), phenylephrine (Phe), and A-61603 (A-61) binding (saturation (sat.) or competition (comp.)), solubilized in 0.1% DDM (sol.) or when overexpressed in COS-7 cells (cells), or with a CRE-based functional assay (CRE). The WT receptors were unstable in solution, and thus no binding could be measured (N.A.). N.D. indicates values were not determined and (-) that curves could not be fitted. Data are the mean \pm SEM of three independent experiments. The symbols *, ‡, †, •, and \perp indicate statistically different pairs of values in each row based on one-way ANOVA with Tukey multiple comparisons tests. IP₁ (Figure 1F). Agonist stimulation of COS-7 cells overexpressing α_{1A} -AR A4 and α_{1B} -AR #15 was also unable to activate a cyclic adenosine monophosphate (cAMP) response element (CRE) reporter gene, which reads out GPCR activation of several pathways^{37,38} (Supporting Information Figure 7D), indicating that α_{1A} -AR A4 and α_{1B} -AR #15 are inactive GPCRs.

A Highly Stabilizing Phe to Leu Mutation Reduces Ligand Affinities. The reduction in agonist affinities and the lack of agonist-induced signaling in cells supported the notion that α_{1A} -AR A4 and α_{1B} -AR #15 preferentially exist in inactive states. We sought to determine, however, if these affinity reductions were due to selected mutations around and in the epinephrine binding sites and extracellular vestibules of α_{1A} -AR A4 and α_{1B} -AR #15. α_{1A} -AR F3 contains all the same mutations as α_{1A} -AR A4 around the binding site except for L80M (2.45x45), which packs behind binding-site residues D106 and F312 (Supporting Information Figure 5). The affinities of phenylephrine and prazosin at α_{1A} -AR F3 were not significantly different to α_{1A} -AR A4 (Supporting Information Figure 8A), suggesting that L80M does not perturb ligand binding affinities. The conservative F86Y substitution at the top of the binding site (2.58x57) in α_{1A} -AR A4 is unlikely to affect ligand-binding affinity, as mutation of this residue to methionine has been shown to have little effect on ligand binding.³⁹ α_{1B} -AR #15 contained two mutations in ECL2, D191Y and E194G, which were found to not cause statistically significant changes to ligand binding affinities when reverted (Supporting Information Figure 8B).

These analyses left a mutation at position 7.39x38 in both subtypes (F312L in α_{1A} -AR A4 and F334L in α_{1B} -AR #15), which is positioned at the top of the epinephrine binding sites. Reversion of this mutation resulted in significant improvements in both phenylephrine (agonist) and prazosin (antagonist) affinities at both subtypes (Figure 2A,B); however, the temperature stabilities of these back mutants were significantly reduced (Figure 2C). Position 7.39 is known to be important for ligand binding to α_{1A} -AR.⁴⁰ This position forms part of both the consensus ligand binding pocket and the consensus inter-TM contact network of class A GPCRs² and makes contacts with 6.51 (phenylalanine in both α_{1A} - and α_{1B} -AR). Mutation of 7.39x38 to leucine in α_{1A} -AR A4 and α_{1B} -AR #15 may stabilize the receptors by strengthening the inter-TM contact network, to the detriment of ligand binding affinity. To ensure no loss of receptor competency during NMR experiments, α_{1A} -AR A4 and α_{1B} -AR #15 were chosen for further study. Since both stabilized receptors contained the same mutation at 7.39x38 and exhibited similar changes to ligand binding affinities, we believe that comparison of their ligand binding characteristics is representative of the two subtypes.

STD NMR Epitope Mapping of Epinephrine Binding to α_{1A} -AR A4 and α_{1B} -AR #15. STD NMR is well suited to the detection of weak ligand—protein interactions with fast dissociation kinetics (K_d range between 100 nM and 1 mM), which leads to a rapid exchange of the ligand between the bound and unbound states, resulting in saturation of more ligand molecules in solution and thus more STD enhancement. Thus, the α_1 -AR agonist epinephrine ($K_i = 290 \pm 28 \text{ nM}^{41}$) was chosen as the initial test ligand (Figure 3A). STD signals were observed for the aromatic protons, the methylene group, and the methyl group of epinephrine upon binding to α_{1A} -AR A4 and α_{1B} -AR #15 (Figure 3). To determine the specificity of these STD signals, similar samples were prepared with the



Figure 2. A mutation at 7.39x38 in α_{1A} -AR A4 and α_{1B} -AR #15 caused reduced ligand affinities but improved receptor stability. (A,B) QAPB competition binding and (C) temperature denaturation assays were performed against purified α_{1A} -AR A4 (black circles and lines), α_{1A} -AR A4 (L312F; gray circles and lines), α_{1B} -AR #15 (dark blue triangles and lines), and α_{1B} -AR #15 (L334F; pale blue triangles and lines). Open symbols and dashed lines in A and B indicate competition with phenylephrine (Phe), whereas solid symbols and lines are from competition with prazosin (Praz). Binding was performed at 25 °C for 2 h to replicate NMR conditions.

addition of the high-affinity α_1 -AR antagonist, prazosin, as a competitor. The addition of prazosin significantly attenuated the STD signals, demonstrating that the detected signals corresponded to specific binding of epinephrine to the orthosteric site in both stabilized α_1 -ARs (Figure 3). Importantly, no STD signals were observed for epinephrine in samples containing the same detergent buffer, but no receptor protein (Supporting Information Figure 9).

STD intensities depend on ligand concentration, ligand dissociation rate, the duration of radio-frequency irradiation, the R_1 relaxation rates of the individual ligand protons, and crucially the relative efficiency by which the protons of the ligand are saturated.²⁹ Considering that the efficiency of saturation transfer is related to the intimacy of contact between a given ligand proton and the receptor, STD NMR can be used to map binding epitopes.⁴² STD NMR build-up curves for epinephrine binding were generated by varying the saturation time and tracking the relative STD intensities of epinephrine protons binding to each receptor (Figure 4A,B). To control for the influence of R_1 relaxation, the initial build-up rates (r_0) of



Figure 3. STD NMR of epinephrine binding to stabilized α_1 -ARs. (A) Chemical structure of epinephrine with assigned protons labeled. (B) ¹H STD NMR spectrum of 500 μ M epinephrine binding to α_{1A} -AR A4. (C–F) STD signals of epinephrine binding to receptors (blue spectra) could be attenuated by adding 10 μ M of high affinity antagonist prazosin (red spectra), in samples containing α_{1A} -AR A4 (C,D) or α_{1B} -AR #15 (E,F). Epinephrine resonances are labeled according to A.

intimately interacting protons were calculated by fitting these curves to eqs 1 and 2 (Methods). The resultant normalized r_0 values revealed that the aromatic hydrogens of epinephrine (labeled 1–3) make the most intimate contact with α_1 -ARs compared to the methyl group (5) and the methylene group (4; Figure 4C), which fits well with the known structure– activity relationship (SAR) of epinephrine binding to α_1 -ARs. The primary binding epitope of epinephrine is known to be the phenyl group and hydroxyl substituents, with removal of one or both hydroxyl groups completely abolishing receptor affinity.⁴³ The meta-hydroxyl substituent of the aromatic ring may make hydrogen bonds with serine 5.42x43 of the receptors. Interaction between aspartate 3.32x32 of α_1 -ARs and the amine of epinephrine also plays a vital role in binding and receptor activation.

Norepinephrine lacks the methyl substitution in the amino group of epinephrine but binds only slightly weaker than epinephrine to α_1 -ARs,⁴¹ indicating that the methyl group is a

minor contributor to receptor binding. Extending the alkyl side chain on the N atom of a catecholamine reduces affinity for α_1 -ARs and produces β -AR selective agonists,⁴³ highlighting that the binding mode of epinephrine is different between α_1 - and β -ARs. As expected, no significant difference in the binding mode of epinephrine was observed between α_{1A} -AR and α_{1B} -AR. Selective α_{1A} -AR agonists are generated by adding bulky substituents at the ortho-2 position of the catechol ring,⁴³ suggesting that the α_{1B} -AR binding site interacts more intimately with this side of the phenyl ring and thus cannot accommodate larger modifications. While not statistically significant, STD build-up analysis indicated that the ortho-2 hydrogen (proton 1 in Figure 3) of epinephrine may interact more intimately with α_{1B} -AR compared to α_{1A} -AR (Figure 4C). Thus, analyses such as these could be useful for medicinal chemistry campaigns aimed at generating selective compounds at closely related receptor subtypes.

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Figure 4. STD build-up analysis of epinephrine binding to (A) α_{1A} -AR A4 and (B) α_{1B} -AR #15. (C) Epitope maps were generated by fitting the curve to a first-order exponential and extracting the initial slope of each build-up curve (r_0 , see Methods), and normalizing these values to the resonance with the highest r_0 at each receptor (% max). Protons are labeled as in Figure 3.

STD NMR Epitope Mapping of A-61603 Binding to α_{1A} -AR A4 and α_{1B} -AR #15. A-61603 is an agonist with 10-100-fold selectivity for α_{1A} -AR over α_{1B} -AR and thus would be expected to bind with a different epitope to α_{1A} -AR compared to α_{1B} -AR. A-61603 retained selectivity for the stabilized α_{1A} -AR over stabilized α_{1B} -AR, despite binding with weaker affinities than reported at WT receptors (Table 2). Specific STD signals could be detected for the hydrogen substituents of the phenol group (labeled 1-2 in Figure 5A, D, and I), the two methylenes of the imidazoline group (labeled 8-9 in Figures 5B, E, and I), the methyl of the methanesulfonamide group (labeled 7 in Figures 5B, E, and I), and the hydrogens of the cyclohexane group (labeled 3-6 in Figures 5B, C, E, F, and I) against both α_{1A} -AR A4 and α_{1B} -AR #15. STD NMR build-up curves were generated for A-61603 at both receptors (Figure 5G and H), and epitope maps were prepared by calculating the initial build-up rate (r_0) for each proton, or proton group, on each receptor (Figure 5J). These data revealed that the methanesulfonamide group of A-61603 binds more intimately to α_{1A} -AR A4 than to α_{1B} -AR #15, whereas the imidazoline group exhibited faster build-up rates upon binding α_{1B} -AR #15

compared to α_{1A} -AR A4. This is consistent with structure– activity studies on A-61603 where modification of the methanesulfonamide group, for example by replacement with propanesulfonamide, leads to a loss of selectivity for α_{1A} -AR.⁴⁴

Epitope maps reveal which ligand atoms are in close contact with the receptor, information which can be used to refine structural models of ligand-receptor complexes. Since no α_1 -AR crystal structures have been solved, a homology model of α_{1A} -AR based on the active-state structures of the β_2 -AR (PDB: 4LDO and 3SN6) was used for in silico ligand docking. Epinephrine and A-61603 were docked into the binding site in this model using Molsoft ICM Pro (Figure 6A and B). In the top five docking poses of A-61603, the methanesulfonamide group projected toward Ser188 in TM5 of α_{1A} -AR (GPCRdb residue 5.42x43; Figure 6B), which is known to provide a crucial hydrogen bond to the meta-hydroxyl group of epinephrine⁴³ (Figure 6A). The imidazoline group of A-61603 thus takes on the role of the amine in epinephrine, interacting with Asp106 (3.32x32), which is also a crucial binding site residue in adrenoceptors.⁴³

To validate the STD epitope map and the proposed A-61603 binding mode in α_{1A} -AR, we sought mutations that would not significantly disrupt the ligand binding sites of both receptors but would subtly change the local environment around the methanesulfonamide group upon binding, which we hypothesize would be reflected in STD experiments. The catecholamine binding sites of α_{1A} -AR and α_{1B} -AR are highly similar, with the only difference in this area being at position 5.43x44, Ala189 in α_{1A} -AR or Ser208 in α_{1B} -AR (Figure 6A,B). Insertion of serine at this position into α_{1A} -AR⁴⁵ or alanine into this position of α_{1B} -AR^{46,47} has been reported to not significantly affect ligand binding or receptor function. Thus, position 5.43x44 in α_{1A} -AR A4 was mutated to serine (α_{1A} -AR A4 (A189S)) and to alanine in α_{1B} -AR #15 (α_{1B} -AR #15 (S208A)). The binding affinities of QAPB, prazosin, and A-61603 were significantly altered at α_{1A} -AR A4 (A189S) compared to α_{1A} -AR A4 (Supporting Information Figure 10, Figure 6C, and Table 2), whereas no differences in ligand binding affinities were observed between α_{1B} -AR #15 (S208A) and α_{1B} -AR #15 (Supporting Information Figure 10, Figure 6D and Table 2). Despite the disrupted binding site in α_{1A} -AR A4 (A189S), STD build-up analyses were performed on epinephrine and A-61603 binding to both α_{1A} -AR A4 (A189S) and α_{1B} -AR #15 (S208A).

Consistent with the ligand binding analysis, the resultant epitope maps for epinephrine and A-61603 at α_{1A} -AR A4 (A189S) were significantly different than at α_{1A} -AR A4 (Figure 6E and F), especially for the phenyl group protons (proton 2 on epinephrine and protons 1 and 2 on A-61603), which based on our docking results, bind close to position 5.43x44 in α_{1A} -AR. It is likely that the addition of serine at 5.43x44 alters the hydrogen bonding network between α_{1A} -AR Ser188, Ser192, and the ligands. Interestingly, for A-61603 binding to α_{1A} -AR A4 (A189S), the initial build-up rate of the methanesulfonamide methyl group was significantly increased, suggesting that this group contacts α_{1A} -AR A4 (A189S) within the vicinity of 5.43x44, as predicted. The STD build-up epitope map of epinephrine binding to α_{1B} -AR #15 (S208A) was not significantly different from that at α_{1B} -AR #15, also consistent with ligand-binding analysis (Figure 6E). However, STD buildup analysis of A-61603 at α_{1B} -AR #15 (S208A) revealed a significant difference in the initial build-up rate of the methanesulfonamide group, compared to that at α_{1A} -AR A4 (Figure 6F). The STD build-up analysis of α_{1A} -AR A4 (A189S)



Figure 5. (A–F) STD signals of A-61603 binding receptors (blue spectra) could be attenuated by adding 10 μ M of high affinity antagonist prazosin (red spectra), in samples containing α_{1A} -AR A4 (A–C) or α_{1B} -AR #15 (D–F). STD build-up curves of A-61603 binding to (G) α_{1A} -AR A4 and (H) α_{1B} -AR #15. A-61603 resonances are labeled according to I, on the structure of A-61603. (J) Normalized initial build-up rates (r_0) from STD build-up curves of A-61603 binding to α_{1A} -AR A4 (red bars) and α_{1B} -AR #15 (blue bars). Asterisks (*) indicate statistically significant differences based on two-way ANOVA and an uncorrected Fisher's Least Significant Difference (LSD) test.

and α_{1B} -AR #15 (S208A) demonstrates that the binding we are observing with STD NMR is reflective of primary, orthosteric binding site interactions. Furthermore, the epitope maps and the docking result against α_{1A} -AR (Figure 6B) strongly suggest that the methanesulfonamide of A-61603 is binding near 5.43x44. Overall, the STD build-up analysis of epinephrine and A-61603 binding to α_{1A} -AR A4 (A189S) and α_{1B} -AR #15 (S208A) support the proposed binding modes at α_{1A} -AR.

The endogenous agonists of most nonpeptide, rhodopsinfamily GPCRs bind with relatively weak, micromolar affinities. To date, however, there are only two micromolar-affinity agonist-bound GPCR structures published, that of the β_2 -AR bound to epinephrine³¹ and that of turkey β_1 -AR bound to dobutamine.³² Thus, there is a need for other methods to probe the atomic-level mechanisms underlying agonist binding to GPCRs, especially to probe binding differences between closely related subtypes. We believe that this study is the first to demonstrate that a combination of STD NMR epitope mapping, homology modeling, docking, and mutational analysis can determine binding modes and selectivity determinants of weak affinity ligands. Ligand-observed NMR methods have been applied to GPCRs before. STD NMR has been used to investigate the binding of a protein ligand to the extracellular domain of the sweet taste receptor³⁰ and cholesterol to the β_2 -AR,¹⁵ but to our knowledge the use of STD NMR to measure orthosteric agonist-GPCR binding, or for GPCR ligand epitope

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Figure 6. (A) Dock of epinephrine (pale blue) into an active-state homology model of α_{1A} -AR. Residues known to play key roles in epinephrine binding (Asp106, Ser188, and Ser192) are labeled from transmembrane domains 3–5 (TM3–5). (B) Overlay of the top 5 docking conformations of A-61603 (pale blue) binding to an active-state homology model of α_{1A} -AR. Protons of interest from the methanesulfonamide (colored green and labeled with 7) and the imidazoline (labeled with 8 and 9) groups in the A-61603 molecules are labeled. (C) QAPB competition binding against purified α_{1A} -AR A4 (solid circles and lines), α_{1A} -AR A4 (A189S; crosses and dashed lines), and in D, α_{1B} -AR #15 (solid circles and lines) and α_{1B} -AR #15 (S208A; crosses and dashed lines). Black symbols and lines are competition with prazosin (Praz). Red symbols and lines are competition with phenylephrine (Phe), and blue symbols and lines are competition with A-61603. (E) Normalized initial build-up rates (r_0) from STD build-up curves of epinephrine binding and (F) A-61603 binding, to α_{1A} -AR A4 (red bars), α_{1A} -AR A4 (A189S) (orange bars), α_{1B} -AR #15 (dark blue bars), and α_{1B} -AR #15 S208A (pale blue bars). Proton signals are labeled according to Figure 3A (epinephrine) and Figure 5A (A-61603). Asterisks indicate statistically significant differences based on two-way ANOVA and an uncorrected Fisher's Least Significant Difference (LSD) test.

mapping, has not been reported. Recently, the application of two other ligand-observed NMR methods to GPCRs has been reported, Transferred Nuclear Overhauser Effect Spectroscopy (Tr-NOESY)²⁶ and INPHARMA,²² both of which can be used to model the structures of weakly binding ligands bound to GPCRs. Finally, an STD-like method called methyl-directed transferred cross-saturation has been applied to detect the binding, and map the binding interface, of the chemokine SDF-1 to its receptor CXCR4 in detergent²⁷ and lipid bilayers;²⁸ however, this method is more suited to monitoring receptor protein ligand interactions and requires the use of isotopically labeled ligands.

 α_1 -ARs are important regulators of cardiac function, vascular blood pressure, and neurotransmission. The high prevalence of cardiovascular diseases today makes α_1 -ARs important drug targets for the treatment of hypertension, but evidence collected from studies using knockout mice and transgenic mice expressing constitutively active mutants of α_{1A} -AR and α_{1B} -AR has revealed that these α_{1} -AR subtypes mediate opposing roles in several other disease models. An example of this is in the heart, where α_{1A} -AR and α_{1B} -AR, expressed in myocytes, play important roles in the progression of diseases such as cardiac hypertrophy and heart failure. α_1 -ARs are primarily responsible for catecholamine-induced cardiac hypertrophy, which can be either beneficial (i.e., adaptive hypertrophy through α_{1A} -AR stimulation) or detrimental, increasing the risk of heart failure (chronic, maladaptive hypertrophy through α_{1B} -AR stimulation) (reviewed in ref 43). While clinically effective subtype-selective drugs are desirable, the high sequence conservation between the two subtypes makes the design of highly subtype-selective drugs challenging. In this study, CHESS was employed to engineer detergent-stable α_{1A} -AR and α_{1B} -AR variants. These detergent-stable α_{1A} -AR and α_{1B} -AR mutants enabled the analysis of specific GPCR-ligand interactions in a purified system using STD NMR. Furthermore, STD build-up experiments at both receptor subtypes were used to generate epitope maps of epinephrine and the α_{1A} -AR-selective agonist, A-61603, revealing the molecular determinants for the selectivity of A-61603. In the absence of interacting G proteins to stabilize active receptor states, this study has predominantly probed agonist binding to inactive receptor states. Thus, we cannot rule out the existence of additional selectivity determinants that only exist in active receptor states.

In summary, this work opens new opportunities for investigating, and screening for, ligand binding to these important membrane proteins and for refining and validating structural models of ligand-GPCR complexes in general. Our approach shows that GPCRs that have been evolved to be sufficiently stable under *in vitro* experimental conditions are valuable tools for medicinal chemistry campaigns aimed at generating selective compounds at closely related receptor subtypes.

METHODS

Bacterial Strains, Cell Lines, and Reagents. The ElectroMAX DH5 α E. coli strain (ThermoFisher Scientific) was prepared as described previously.³³ The E. coli C43 (DE3) strain was purchased from Lucigen Corporation. COS-7 African green monkey kidney cells (ATCC CRL-1651) were a kind gift from Dr. Wah Chin Boon (The Florey Institute of Neuroscience and Mental Health). The expression cassette comprising the custom bacterial expression vector pDS11 was synthesized by Genscript Corporation and contained the following sequences in frame: maltose binding protein (MBP) signal peptide, His10 tag, MBP, Asp10 linker, human Rhinovirus 3C protease site, receptor cloning site (BamHI - NheI), 3C protease site, 2 x GGGS linker, mCherry, and Avi-tag. This cassette was subcloned into the expression vector pIQ (pQE-30 from Qiagen corporation, containing the lac repressor encoding gene Lacl⁴⁸). The B. subtilis sacB gene was inserted between the BamHI and NheI restriction sites to generate pDS11. Human α_{1A} - and α_{1B} -ARs encoding genes in mammalian expression vector pcDNA3.1 were purchased from cDNA Resource Center (www.cdna.org). *n*-Dodecyl β -D-maltoside (DDM), *n*-decyl β -D-maltoside (DM), and cholesteryl hemisuccinate (CHS) were purchased from Anatrace. Prazosin, epinephrine, phenylephrine, and A-61603 were purchased from Sigma-Aldrich. BODIPY-FL-prazosin (QAPB [Quinazoline Piperazine Bodipy]) was purchased from ThermoFisher Scientific.

Stabilization of α_{1A} - and α_{1B} -AR with CHESS. Error-prone libraries were generated as described previously,³³ using libraries preselected for high expression in E. coli as starting material.⁴⁹ First, 50 mL cultures expressing the receptor mutant libraries were encapsulated as described previously³³ and resuspended into 4 mL selection buffer (10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 137 mM NaCl, 2.7 mM KCl, 1 mM EDTA, 50 µg/mL kanamycin at pH 7.4) supplemented with detergents (either 1% DDM, 1.7% DM, or a mixture of 1% DDM, 0.5% CHAPS, and 0.1% CHS) for 3 h. Microcapsules were treated with 200 nM QAPB to fluorescently identify stable mutant-containing capsules. The most fluorescent microcapsules (top 0.5 to 1.0% of the QAPB positive populations) were sorted using a FACS ARIA III (BD Biosciences). PCR was used to extract the DNA sequences of selected clones, and sorted populations were recloned, expressed, encapsulated, and sorted until stable receptors were isolated, as described in Supporting Information Figures 1 and 2. From the final selected populations, 47 α_{1A} -AR individual clones and 23 α_{1B} -AR individual clones were expressed and the receptors solubilized as described previously.33 Detergent-solubilized proteins were immobilized onto Streptavidin T1 Dynabeads (ThermoFisher Scientific) and assayed for ligand binding as described previously.^{33,50} After incubation in detergents, QAPB (485/12 and 520 nm excitation/emission) and mCherry (544 and 590/10 nm excitation/emission) fluorescence was

measured using a POLARstar OMEGA plate reader (BMG Labtech). Receptors were purified and characterized as described in the Supporting Information and Methods.

IMAC Purification of α_{1A} -AR A4 and α_{1B} -AR #15. α_{1A} -AR A4 and α_{1B} -AR #15 were transformed into E. coli C43 (DE3) and expressed in 1 L cultures as described previously.⁴⁹ Harvested cells were washed once with 30 mL of phosphate buffer (50 mM potassium phosphate, 150 mM NaCl, pH 8.0), then resuspended in 20 mL of IMAC solubilization buffer (1% DDM, 0.5% CHAPS, 0.12% CHS, 1 mg mL⁻¹ lysozyme, 0.1 mg mL⁻¹ DNaseI, Roche cOmplete protease inhibitor in phosphate buffer) and sonicated for 15 cycles with 10 s on and 20 s off at 30% power at 4 °C. An additional 30 mL of IMAC solubilization buffer was added to the sonicated cells and mixed at 4 °C for 2 h. Cell lysates were clarified by centrifugation at 10 000 rpm at 4 °C for 30 min followed by passing through a 0.45 μ m syringe filter. A total of 1.5 mL of TALON metal affinity resin (Clontech) equilibrated with 10 column volumes (CV) of equilibration buffer (0.05% DDM in phosphate buffer) was added to the clarified lysate and mixed gently for 2 h at 4 °C to capture full-length α_1 -ARs fusion proteins. The resin was subsequently washed with 30 CV of IMAC wash buffer (0.05% DDM, 50 mM potassium phosphate, 500 mM NaCl, 10 mM imidazole, pH 8.0), and receptors were eluted with 10 CV of IMAC elution buffer (0.05% DDM, 50 mM potassium phosphate, 500 mM NaCl, 300 mM imidazole, pH 8.0). Elutions were pooled and concentrated to 1 mL using Amicon Ultra-15 100 kDa molecular weight cutoff (MWCO) centrifugal concentrators (Merck Millipore). Buffer was exchanged (0.05% DDM, 50 mM potassium phosphate, 100 mM NaCl, pH 7.4) using PD-10 desalting columns (GE Healthcare Life Sciences) and concentrated again with Amicon Ultra-15 100 kDa MWCO centrifugal concentrators to a final volume of 0.4 mL. Protein concentration was determined by Direct Detect (Merck Millipore). Deuterated glycerol was added to 20%, and aliquots of the proteins were snap-frozen in liquid nitrogen and stored at -80 °C for thermostability, binding assays and STD NMR.

Thermostability of Stabilized α_1 -ARs. To measure apo-state thermostability, 1 nmol of IMAC-purified receptor was resuspended in 2.5 mL of 0.1% DDM in PBS. A total of 100 μ L of this solution was aliquoted into 24 wells of a 96-well PCR plate. Using a gradient thermocycler, 10 duplicates from the 24 wells were heated for 30 min at 20 to 50 °C. Two remaining duplicates were left at 4 °C for normalization. A total of 90 μ L of each sample was transferred to a KingFisher 96-DeepWell plate with each well containing 2 μ L paramagnetic Dynabeads (His-Tag Isolation, ThermoFisher Scientific). Receptor capture (1 h incubation), ligand binding (2 h incubation), and washing of unbound ligands (1 min incubation) were automated using a KingFisher 96 magnetic particle processor. For ligand binding, 100 nM QAPB was used in PBS and 0.1% DDM. To determine nonspecific binding, 50 μ M prazosin was added to this buffer. After binding, beads were then washed once in 200 μ L of PBS and 0.1% DDM followed by resuspension in 100 μ L of PBS, 0.1% DDM, and 300 mM imidazole to elute the receptors from the beads. A total of 90 µL of this was transferred to a 96-well Greiner Bio-One nonbinding black plate. Fluorescence of QAPB binding at each temperature point was measured using a POLARstar OMEGA plate reader and normalized to 0% and 100% maximum binding using 4 °C with and without unlabeled prazosin competition, respectively. Apparent $T_{\rm m}$ values were determined by fitting the temperature denaturation curves with asymmetrical sigmoidal nonlinear regression in Graphpad Prism 6. Similarly for ligand-bound thermostability, receptors were first preincubated with 100 nM QAPB for 2 h at 4 °C before heat treatment from 23.3 to 60 °C on a gradient thermocycler. Samples were captured onto beads for 1 h, washed, and eluted similarly to the above steps. Data are represented as mean \pm SEM performed in three independent experiments.

Saturation and Competition Binding on Stabilized α_1 -ARs. A total of 1 nmol of IMAC-purified receptors was resuspended in 5 mL of 0.1% DDM in PBS and immobilized onto 200 μ L of Dynabeads (Streptavidin T1) for 30 min at 4 °C. A total of 100 μ L of receptor immobilized beads suspension was aliquoted into 48 wells on a 96-DeepWell plate, and immobilized receptors were transferred to

another 96-DeepWell plate for saturation and competition binding using a KingFisher Flex magnetic particle processor. For saturation binding, immobilized receptors on each well were incubated with 100 μ L of 0.1% DDM in PBS containing 0.075 to 50 nM of QAPB for 2 h at 4 or 25 °C. Nonspecific binding was determined by repeating the experiment in the presence of 10 μ M of prazosin. For competition binding, immobilized receptors on each well were incubated with 100 μ L of 0.1% DDM in PBS containing 10 nM QAPB with the addition of 0.2 to 10000 μ M phenylephrine, or 0.02 to 1000 μ M epinephrine, or 0.02 to 1000 nM prazosin, or 1 to 2000 µM A-61603 for 2 h at 4 or 25 °C. Immobilized receptors were washed with 200 µL of PBS and 0.1% DDM and resuspended in 100 μ L of PBS and 0.1% DDM. A total of 90 μ L of resuspended immobilized receptors was transferred to a 96-well nonbinding black plate, and normalized QAPB/mCherry binding was measured similar to the above. Data are represented as mean ± SEM performed in triplicate from three independent experiments.

Characterization of Stabilized α_1 -ARs Expressed in Mamma**lian Cells.** The C-terminal tails of the α_{1A} - and α_{1B} -ARs were cloned back into the stabilized receptors using an overlap PCR approach. COS-7 fibroblast-like cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS, 1% L-glutamine, and 1% penicillin/streptomycin (Life Technologies, California, USA) at 37 °C with 95% O₂/5% CO₂ and 85% humidity. Before transfection COS-7 was plated on 96-well Viewplates (PerkinElmer, Massachusetts, USA) at 2.0×10^5 cells in 100 μ L of medium and incubated overnight for 50 to 70% confluency the next day. For each well, 0.25 μ g of pcDNA3.1 expression vector containing no receptor, WT, or stabilized α_1 -ARs and 0.5 μ L of Lipofectamine 2000 (Life Technologies) were each added to 12.5 µL of Opti-MEM (Life Technologies) in two separate 1.5 mL microfuge tubes. After 5 min of incubation, both tubes were mixed together and incubated for a further 30 min. A total of 25 µL of Lipofectamine2000/DNA mix was added onto the well and incubated for 24 h. Saturation binding assays using [³H]-prazosin were performed on transfected cells. Media were aspirated, and the transfected COS-7 cells were washed with 200 μ L of PBS before radioligand binding. Cells were incubated with 100 μ L of HEM buffer (20 mM HEPES, 1.4 mM EGTA, 12.5 mM MgCl₂, 150 mM NaCl, pH 7.4) containing 0.025 to 10 nM [³H]-prazosin in triplicate for 1 h at RT. Cells transfected with WT or stabilized α_1 -ARs were incubated with 100 μ L of HEM buffer containing 0.25 to 50 nM [³H]-prazosin in triplicate for 1 h at RT. Nonspecific binding was determined by repeating the experiment in the presence of 100 μ M Phentolamine (a neutral α -AR antagonist⁴¹). Cells were then washed with 200 μ L of PBS and solubilized with 50 μ L of 0.2 M NaOH for 10 min. A total of 100 µL MicroScint-20 (PerkinElmer) was added to solubilized cells and incubated for 30 min in the dark before measurement on a Wallac Microbeta Counter (PerkinElmer) for 1 min. Data were represented as mean ± SEM performed in triplicate from three independent experiments. Statistical analysis was performed using one-way ANOVA with statistical significance valued at P < 0.05.

Signaling of the α_{1A} - and α_{1B} -ARs was assessed using the IP-One HTRF assay kit (Cisbio Bioassays), to measure the IP₁ (inositol 1-monophosphate) accumulation, following the manufacturer's protocol. Stimulation of the cells was performed at 37 °C in a CO₂ incubator for 2 h. Time-resolved fluorescence was measured at 665 and 620 nm emission wavelengths (330 nm excitation), and the 665/620 emission ratios were calculated. A standard curve was plotted and fitted using the nonlinear sigmoidal function in Graphpad Prism 6 to determine the concentration of IP₁. Data were represented as mean ± SEM performed in triplicate in three to four independent experiments. Statistical analysis was performed using one-way ANOVA with statistical significance valued at P < 0.05.

To assess signaling by the activation of CRE-induced expression of the β -galactosidase reporter, WT α_{1A^-} and α_{1B} -AR stably expressing COS-7 cells were transfected with a β -galactosidase expression plasmid under the control of the CRE promoter and assayed as described previously.⁵¹ For stimulation, DMEM medium was supplemented with 0.1 nM to 250 μ M phenylephrine or 5 μ M forskolin for 6 h at 37 °C in a 5% CO₂ incubator. Data were normalized to the response induced by 5 μ M or 10 μ M forskolin (100%) and blank (0%) and analyzed using Graphpad Prism 6. Data represented were mean \pm SEM from four independent experiments conducted in triplicate.

NMR Sample Preparation. Stock solutions of 10 mM epinephrine dissolved in 0.1 M HCl were supplemented with 1 mM ascorbic acid to prevent oxidation. A-61603 was dissolved in water to 10 mM. Stock solutions of 5 mM prazosin were prepared in deuterated methanol. STD NMR samples constituted 5 μ M receptor and 500 μ M test ligands with and without competition of 10 μ M prazosin in 500 μ L of DDM buffer (0.05% (or 1 mM) DDM, 50 mM potassium phosphate, 100 mM NaCl, 10% ²H₂O, pH 7.4) in 5 mm NMR tubes. For STD NMR with 500 μ M epinephrine, the buffer was supplemented with 1 mM ascorbic acid. For the acquisition of build-up STD spectra, 1 mM CHS and 2 mM CHAPS were added to both α_{1a} -AR A4 and α_{1B} -AR #15 to improve the receptor stability.

NMR Spectroscopy. 1D ¹H STD spectra were acquired at 25 °C on a 700 MHz Bruker Avance IIIHD spectrometer using a cryogenically cooled triple resonance probe. Data were acquired with saturation pulses ranging from 0.25 to 5 s duration, using a train of 50 ms Gaussian pulses with a B1 field of about 130 Hz, separated by 4 μ s delays.⁵² The on- and off-resonance frequencies were -1 and 55 ppm, respectively. To suppress residual protein and water signals, a spin-lock pulse of 40 ms and excitation sculpting were employed, respectively. The relaxation delay between transients was set to 5.1 s and included the saturation pulse. A total of 512 transients were averaged over 32 000 data points and a spectral width of 16 ppm. Prior to Fourier transformation, data were multiplied by an exponential function with 2 Hz line-broadening and zero-filled once. Epinephrine and A-61603 assignments were confirmed in two-dimensional (2D) ¹H total correlation spectroscopy (TOCSY) and Nuclear Overhauser Effect Spectroscopy (NOESY) spectra acquired under the same solution conditions as the STD experiments and in the presence of receptor. Data were analyzed using Mnova NMR (Mestrelab).

STD Buildup Analyses. For build-up series, maximum STD peak intensities were extracted for each saturation time to calculate STD-Amplification Factors (STD-AFs).²⁹ STD-AFs were plotted and fitted using Graphpad Prism 6 to eqs 1 and 2 to calculate the initial build-up rates (r_0) for each resonance of interest. In eqs 1 and 2, STD is the STD signal intensity of a given proton at saturation time t, STD_{max} is the maximal STD or plateau of the build-up curve, and k_{sat} is the observed saturation rate constant.

$$r_0 = \frac{d(\text{STD}_{\text{max}})}{dt(0)} = k_{\text{sat}} \times \text{STD}_{\text{max}}$$
(1)

$$STD = STD_{max}(1 - e^{(k_{sat}t)})$$
(2)

Statistical analysis was performed using two-way ANOVA and uncorrected Fisher's LSD tests with statistical significance defined as P < 0.05.

Docking. The five human α_{1A} -AR homology models on the I-TASSER GPCR-HGmod database (https://zhanglab.ccmb.med. umich.edu/GPCR-HGmod)⁵³ were prepared for docking in Molsoft ICM Pro 3.8 (Molsoft, LLC) by adding missing hydrogens and minimizing all side chains. ICM Pro has been extensively validated for docking small molecules to many protein structures, including GPCRs.⁵⁴ The binding site was defined by a 13 Å by 13 Å by 13 Å cube with the well-established residues comprising the epinephrine binding site (Ser188, Ser189, and Asp106) at its base. Docking of epinephrine was performed at all five homology models using fully flexible ligand and rigid receptor settings in ICM Pro. The top 10 conformations (based on ICM scoring) from each model were inspected for compatibility to the known structure-activity relationship (SAR) of epinephrine at α_{1A} -AR and the crystal structure of epinephrine bound to β_2 -AR (PDB: 4LDO³¹). From these, docking to the first, lowest energy I-TASSER α_{1A} -AR model gave the highest number of realistic epinephrine docking conformations (8 out of 10), and thus this model was used for A-61603 docking, which was performed in the same way as above.

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ASSOCIATED CONTENT

S Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschembio.8b00191.

Supporting Figures 1–10 (PDF)

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Notes

The authors declare no competing financial interest.

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