Extracellular Surface Residues of the $\alpha_{1B}$-Adrenoceptor Critical for G Protein–Coupled Receptor Function

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Received July 1, 2014; accepted October 27, 2014

ABSTRACT

Ligand binding and conformational changes that accompany signaling from G protein–coupled receptors (GPCRs) have mostly focused on the role of transmembrane helices and intracellular loop regions. However, recent studies, including several GPCRs cocrystallized with bound ligands, suggest that the extracellular surface (ECS) of GPCRs plays an important role in ligand recognition, selectivity, and binding, as well as potentially contributing to receptor activation and signaling. This study applied alanine-scanning mutagenesis to investigate the role of the complete ECS of the $\alpha_{1B}$-adrenoceptor on norepinephrine (NE) potency, affinity, and efficacy. Half (24 of 48) of the ECS mutations significantly decreased NE potency in an inositol 1-phosphate assay. Most mutations reduced NE affinity (17) determined from $[^3H]$prazosin displacement studies, whereas four mutations at the entrance to the NE binding pocket enhanced NE affinity. Removing the influence of NE affinity and receptor expression levels on NE potency gave a measure of NE efficacy, which was significantly decreased for 11 of 48 ECS mutants. These different effects tended to cluster to different regions of the ECS, which is consistent with different regions of the ECS playing discrete functional roles. Exposed ECS residues at the entrance to the NE binding pocket mostly affected NE affinity, whereas buried or structurally significant residues mostly affected NE efficacy. The broad potential for ECS mutations to affect GPCR function has relevance for the increasing number of nonsynonymous single nucleotide polymorphisms now being identified in GPCRs.

Introduction

G protein–coupled receptors (GPCRs) regulate major physiologic functions by coupling extracellular stimuli from hormones and neurotransmitters and sensory stimuli from light, odorants, and flavors to intracellular signaling (Rosenbaum et al., 2009). The structure function of GPCRs are regulated by highly conserved motifs in the transmembrane helices (TMHs), including the "ionic lock" between the highly conserved E/DY motif on TMH3 and a glutamate residue on TMH6, the NXXXY motif at the cytoplasmic end of TMH7, and the "rotamer toggle switch" tryptophan in TMH6, which influence receptor transitions between the active and inactive states (Kobilka and Deupi, 2007; Rosenbaum et al., 2009; Granier and Kobilka, 2007; Venkatakrishnan et al., 2013). In addition, the recent crystal structure of the agonist-bound active state of the $\beta_2$-adrenoceptor ($\beta_2$-AR) coupled to $G_s$ reveals dynamic aspects of the cytoplasmic loops that are also critical for GPCR function (Rasmussen et al., 2011). Recent structures of active state GPCRs, including the $\beta_2$-AR, rhodopsin, and M2 muscarinic acetylcholine receptor (Rasmussen et al., 2011; Rosenbaum et al., 2011; Standfuss et al., 2011; Kruse et al., 2013; Venkatakrishnan et al., 2013), suggest that the extracellular loops (ECLs) also contribute to ligand affinity and activation mechanisms, whereas the role of the extracellular surface (ECS) residues on GPCR structure function has been largely overlooked (Nobles et al., 2011; Hu et al., 2013).

The $\alpha_{1B}$-adrenoceptors ($\alpha_{1B}$-ARs) belong to class A GPCRs, the largest and most extensively characterized GPCRs of the rhodopsin-like receptor family. The growing number of crystal structures of bovine rhodopsin (Palczewski et al., 2000; Standfuss et al., 2011), bovine opsin (Park et al., 2008; Scheerer et al., 2008), human $\beta_2$-adrenergic (Cherezov et al., 2007; Rasmussen et al., 2011; Rosenbaum et al., 2011; Venkatakrishnan et al., 2013), turkey $\beta_1$-adrenergic (Warne et al., 2008), human $\alpha_{2A}$-adrenoine (Jaakola et al., 2008), human dopamine D3 (Chien et al., 2010), and human muscarinic M2 (Haga et al., 2012; Kruse et al., 2013) and rat M3 (Kruse et al., 2012) receptors now allow rational structure-based drug design and accurate modeling of other class A GPCRs (Katritch et al., 2012; Matsoukas et al., 2013; Ragnarsson et al., 2013; Rodriguez and Gutierrez-de-Teran, 2013; Shim et al., 2013; Stevens et al., 2013). However, except for a conserved disulfide bond that connects a cysteine residue in ECL2 and a cysteine residue located at the extracellular end of TMH3, which is critical for receptor folding and cell surface expression (Zeng et al., 1999), ECLs are poorly considered.

This work was supported by an Australian National Health and Medical Research Council (NHMRC) Project [Grant 011246], an NHMRC Program [Grant 569927], and an NHMRC Principal Research Fellowship [Grant 1019761].

dx.doi.org/10.1124/mol.114.094557.

This article has supplemental material available at molpharm.aspetjournals.org.
(Vaidehi et al., 2014). For example, ECL2 in rhodopsin forms a short β-sheet that caps the covalently bound 11-cis-retinal and ECL2 in β1- and β2-ARs forms a short α-helix, whereas ECL2 in the A2AR-adenosine receptor lacks a defined secondary structure. Our model of the hamster α1B-AR, which was built from the crystal structure of the turkey β1-AR (Protein Data Bank code 2VT4) (Warne et al., 2008), predicts a highly ordered ECL2, with several intramolecular interactions, a salt bridge, and a conserved disulfide bond, but no well defined secondary structure (Ragnarsson et al., 2013). Interestingly, breaking this structurally important disulfide bond facilitated binding of the α1B-AR selective allosteric antagonist ρ-conotoxin-TIA, despite reducing norepinephrine (NE) potency, affinity, and efficacy (Ragnarsson et al., 2013).

Mechanistic insight into how conformational changes in the ECS can influence movement in the TMHs and thus impact on activation and intracellular signaling is important to fully understand the GPCR activation process and will facilitate the rational design of state-dependent GPCR modulators. Recent NMR studies of rhodopsin activation have shown that ECL2 is displaced from the retinal binding site as a consequence of re-arrangements in the hydrogen-bond network connecting ECL2 with the extracellular ends of TMH4, TMH5, and TMH6. Together with a movement of TMH5, which breaks the highly conserved ionic lock (E/DRY), these conformational shifts cause receptor activation (Ahuja et al., 2009). In addition, NMR studies of β2-AR have revealed that agonists and antagonists stabilize distinct GPCR conformations, demonstrating conformational coupling between the ECS and the orthosteric binding site (Bokoch et al., 2010). To investigate in detail how the ECS contributes to GPCR function, we systematically mutated all ECS residues of the α1B-AR to alanine. This study revealed a surprisingly broad contribution to function, with the majority of residues in ECL1 and ECL2 affecting NE potency and/or affinity at the α1B-AR.

Materials and Methods


Transient Expression of α1B-ALs and Membrane Preparation. The transient expression of α1B-ALs and the membrane preparations were performed as described previously (Ragnarsson et al., 2013). To assess the influence of expression levels on NE potency, we transfected WT α1B-AR with reduced DNA levels (0.3, 0.1, and 0.03 of our standard 6 μg DNA per T25 flask) and determined NE half-maximal excitatory concentration (EC50), maximum agonist response (Emax), and KA using the inositol 1-phosphate (IP1) homogeneous time-resolved fluorescence (HTRF) assay, and Bmax from bound levels of a Kd concentration of [3H]prazosin (0.5 nM).

Radioligand Binding Assays. Saturation-binding experiments were performed as described before to determine Bmax using previously determined prazosin Kd at each of the mutants (Ragnarsson et al., 2013). Briefly, membranes from α1B-AR-transfected COS-1 cells (5 μg protein) were incubated with increasing concentrations of [3H]prazosin (5 pM–5 nM) for 60 minutes at room temperature. Nonspecific binding was determined in the presence of 10 μM phentolamine. The affinity of NE at the α1B-AR mutants were determined using the radiolaabeled α1-AR antagonist [3H]prazosin (0.5 nM) or [125I]–β-(iodo-4-hydroxyphenyl)-ethyl-aminomethyl-tetrane (70 pM) for the C195A mutant. Reactions containing radioligand, membranes from α1B-AR–transfected COS-1 cells (5 μg protein), and eight NE concentrations (1 nM–10 nM) in an HEM buffer (20 mM HEPES, 1.5 mM EGTA, 12.5 mM MgCl2, pH 7.4) were established in clear round bottom 96 well plates. Each experiment was performed in triplicate in a total reaction volume of 150 μl. After incubation for 60 minutes at room temperature, the membranes were harvested onto Whatman GF/B filters (PerkinElmer, Waltham, MA) pretreated with 0.6% polyethyleneimine using a Tomtec harvester. BetaPlate scintillant solution (PerkinElmer) was added, and the filter-bound radioactivity was measured using a Wallac MicroBeta (PerkinElmer).

IP1 HTRF Assay. The IP1 HTRF assay was performed as previously described (Ragnarsson et al., 2013).

Molecular Modeling. A molecular homology model of the α1B-AR (Supplemental Material) was built using the crystal structure of the turkey β1-adrenoreceptor (Protein Data Bank code 2VT4) as previously described (Ragnarsson et al., 2013).

Statistics and Data Analysis. Sigmoidal curves for the calculation of EC50 were fitted to individual data points by nonlinear regression using the software package Prism (GraphPad Software, San Diego, CA). Emax was calculated as the difference between the maximal and minimal response to NE and presented as percent of maximal WT response on the day of the assay for normalization. The NE signaling efficacy (NE efficacy) was calculated as the NE pEC50 value minus the NE Kd value, with additional adjustment for the significantly reduced expression levels (observed only for the C118A mutant). Bmax values determined from two 12-point saturation binding experiments with 95% confidence intervals overlapping WT values were considered not significantly different from WT; otherwise, these experiments were performed in triplicate.

An operational model was fitted to the NE concentration response curves for the IP1 accumulation assay to examine the influence of four levels of α1B-AR expression on NE EC50 affinity and efficacy using Prism (GraphPad Software). The operational model generates a global estimate of the functional dissociation constant (Kd) for NE. This model also takes into consideration agonist efficacy (τ), the inverse of the fraction of receptors occupied by NE that produce the half-maximal response, i.e., τ = 10 indicates only 10% of the receptors need to be activated to produce a half-maximal response.

For multiple comparisons, one-way analysis of variance was used with post hoc t tests performed by Dunnett’s method using Prism (GraphPad Software). Values of P < 0.05 were considered significant. The analysis of variance on EC50, Kd, and efficacy data was performed on the log values.

Results

Prazosin Affinity and Bmax at ECS Mutants of the α1B-AR. Prazosin binds in the orthosteric pocket below the ECS of the α1B-AR, and was used to evaluate the expression levels and structural integrity of the 48 ECS mutants (see Fig. 1; Ragnarsson et al., 2013). In ECL1, G109A and G114A showed a significant 9- and 8-fold increase in prazosin affinity (Kd) compared with WT, as reported previously (Ragnarsson et al., 2013). There was no change in prazosin Kd for the mutants in the TMHs adjoining ECL1, TMH2, and TMH3. In ECL2, no mutants significantly affected prazosin...
with the WT receptor (EC50 mutants (3-, 11-, 12-, 16-, and 10-fold, respectively) compared with WT (33-, 26-, 13-, 5-, and 20-fold, respectively, compared with the WT receptor (Fig. 2; Table 1). In contrast, only the P326A mutation in ECL3 caused a small but significant 3-fold change in NE potency compared with WT. In the adjoining TMH6, only the G317A mutant decreased NE potency 4-fold compared with WT, and no changes were observed for mutations in TMH7 (Fig. 2; Table 1).

**Characterization of the Efficacy of NE at ECS Mutants of the α1B-AR.** \( E_{\text{max}} \) to NE was determined at the EC50 mutants and compared with the WT α1B-AR response to identify any influence on maximal signaling induced by NE. Only one of the alanine mutants constructed (C118A α1B-AR) significantly altered the maximal signalling induced by NE, reducing responses to 53% of WT (Table 1). We saw no indication of any increased basal activities for any of the mutants tested that would be indicative of a constitutively active mutant.

**NE Affinity at ECS Mutants of the α1B-AR.** NE affinity \( (K_i) \) was determined from displacement of [3H]prazosin binding for all the ECS mutants to evaluate their effect on NE binding (Fig. 3; Table 1). In ECL1, NE had significantly decreased affinity at the W111A and R115A receptor mutants (6- and 2-fold, respectively), whereas the G109A receptor mutant had 3-fold increased NE affinity compared with the WT receptor \( (K_i = 19.50 \pm 1.83 \mu M; n = 10) \) (Fig. 3; Table 1). In the adjoining TMHs, the α1B-AR mutant V107A in TMH2 increased the NE affinity 2-fold compared with WT, whereas the C118A mutant in TMH3 decreased the NE affinity 26-fold compared with WT (Fig. 3; Table 1).

In ECL2, NE had significantly decreased affinity at the W184A, K185A, E186A, N190A, D191A, D192A, C195A, T198A, and E200A mutants compared with WT (18-, 9-, 6-, 4-, 3-, 2-, 176-, 11-, and 5-fold change, respectively). The P180A, L181A, and G183A mutants in TMH4 adjacent to ECL2 decreased NE affinity (22-, 3-, and 123-fold, respectively) compared with WT, whereas NE potency was unaffected by ECL2-adjoining residues in TMH5 (Fig. 3; Table 1).

In ECL3, only the L323A mutation in ECL3 caused a significant 2-fold decrease in NE affinity compared with the WT receptor. Of the adjoining ECS residues in TMH6, only the G317A mutant decreased NE affinity 3-fold compared with WT, whereas the C118A and K311A mutants in the ECS of TMH7 increased NE affinity (3- and 9-fold, respectively) compared with WT (Fig. 3; Table 1).

**NE Signaling Efficiency of α1B-AR ECS Mutants in Response to NE.** To characterize how effectively NE activated WT and mutant receptors, we removed the influence of NE signaling efficiency \( (E_{\text{max}}) \) to NE was determined at the ECS mutants and compared with the WT α1B-AR (Fig. 3; Table 1). In ECL1, NE had significantly decreased potency at the W111A and R115A receptor mutants (6- and 2-fold, respectively), whereas the G109A receptor mutant had 3-fold increased NE affinity compared with the WT receptor \( (K_i = 19.50 \pm 1.83 \mu M; n = 10) \) (Fig. 3; Table 1). In the adjoining TMHs, the α1B-AR mutant V107A in TMH2 increased the NE affinity 2-fold compared with WT, whereas the C118A mutant in TMH3 decreased the NE affinity 26-fold compared with WT (Fig. 3; Table 1).

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In ECL3, only the L323A mutation in ECL3 caused a significant 2-fold decrease in NE affinity compared with the WT receptor. Of the adjoining ECS residues in TMH6, only the G317A mutant decreased NE affinity 3-fold compared with WT, whereas the F330A and K331A mutants in the ECS of TMH7 increased NE affinity (3- and 9-fold, respectively) compared with WT (Fig. 3; Table 1).
Influence of Expression Levels on NE Potency. To establish the influence of receptor density on NE pharmacology, we transfected with four different levels of WT α1B-AR and fitted to an operational model to the NE concentration response curves from the IP$_{1}$HTRF assay data using Prism (GraphPad Software) (Fig. 5). The operational model gave a global measure of NE functional affinity (K$_{A}$ = 6.94 ± 0.33 M; n = 3). We also plotted log($\tau$) against the corresponding log ($B_{max}$) values, which were significant ($r^2 = 0.67$) and fitted by a linear regression with a slope of 1.8 ± 0.4 (Fig. 5 inset). These data confirm that the levels of WT α1B-AR expression used in this study generated maximal NE responses ($\tau > 10$). NE affinity determined from the IP$_{1}$ experiments using the operational model was significantly lower (>100-fold) than the K$_{A}$ measured from displacement of [3H]prazosin binding to cell membrane preparations (K$_{A}$ = 4.71 ± 0.09 M; n = 10). [3H]prazosin displacement studies performed on intact whole cells expressing α1B-AR showed comparable NE affinity to the binding data obtained from membranes made from transfected cells (K$_{A}$ = 4.09 ± 0.16 M; n = 4).

Discussion

To investigate how ECS residues contribute to function in class A GPCRs, we performed a systematic alanine scan of the complete ECS of the α1B-AR (Fig. 1) and identified residues
that contributed to NE potency, affinity, and/or signaling efficacy. This study revealed that the majority of ECL1 and ECL2 residues and one ECL3 residue, plus a number of ECS residues associated with TMHs, contributed significantly to a1B-AR affinity and/or efficacy (Fig. 6). Many of the functionally significant ECS residues either lined the entrance to the NE binding pocket, where they might affect NE access, or lay outside the entrance, where they might allosterically modulate NE function (Fig. 7). Together, these data support the view that the ECS of GPCRs not only provides a path to the ligand binding pocket but also contributes to receptor activation (Ahuja et al., 2009; Ring et al., 2013).

Previous studies have revealed a number of ECL1 residues that influence receptor activation (Hawtin et al., 2006; Clark et al., 2010; Peeters et al., 2011), including the structurally important WXFG motif common to class A GPCRs (Klco et al., 2006). The a1B-AR has a related WVLG motif in ECL1, where the W111A mutant reduced NE potency without affecting efficacy, whereas the L113A and G114A reduced efficacy without affecting potency. In addition, the adjoining ECL1 mutant Y110A also reduced potency but not efficacy, whereas R115A reduced NE affinity but not efficacy. These residues are positioned outside the entrance to the NE binding pocket, consistent with the WVLG motif playing a structural role in the a1-adrenoceptor. ECL1 adjoins TMH3 and shares conserved interhelical interfaces with all other TMHs, except THM1 and TMH7. ECL1 is thus considered critical in maintaining the structural scaffold of class A GPCRs (Venkatakrishnan et al., 2013), and mutations in ECL1 that affect affinity and efficacy presumably distort ECS confirmation and influence this equilibrium. This is supported by data from the C118A mutation in TMH3, which breaks the disulfide bond to C195 in ECL2, leading to decreased expression and the largest reductions in NE potency arising from reductions in both NE affinity and efficacy. In addition, ECL2 in our model is connected to ECL1 via two hydrogen

![Fig. 2. Effect of a1B-AR mutants on NE EC_{50} in response to NE. Comparison of NE EC_{50} values for WT and a1B-AR mutants to stimulate IP_{3} accumulation in response to increasing concentrations of NE in transiently transfected COS-1 cells. Values are means ± S.E.M. of 38 separate experiments for WT and three to four separate experiments for each mutant (each performed in triplicate). Determining the E_{max} values for IP_{3} accumulation by a1H-AR mutants revealed that only the C118A mutant significantly reduced E_{max} versus the WT receptor (see Table 1). *Significant mutant effects compared with WT receptor.](image1)

![Fig. 3. Effect of a1B-AR mutants on NE K_{i} in response to NE: comparison of NE K_{i} values for WT and a1B-AR mutants. The affinity of NE at the WT receptor and a1B-AR mutants was determined from displacement of the radiolabeled a1-AR antagonist [3H]prazosin (0.5 nM) or [125I]-β-(iodo-4-hydroxyphenyl)-ethyl-aminomethyl-tetralone (70 pM) for the C195A mutant using membranes from a1B-AR–transfected COS-1 cells (5 mg protein) and increasing concentrations of NE. Values are means ± S.E.M. of 10 separate experiments for WT and two to three separate experiments for each mutant (each performed in triplicate). *Significant mutant effects compared with WT receptor.](image2)
bonds (W111 main chain to K193 main chain and R115 main chain to D192 side chain), which might contribute to the influence of these ECL1 mutations on NE affinity.

ECL2 is the most extensively studied and least conserved extracellular loop in GPCRs, both in terms of sequence and structure. In rhodopsin, ECL2 folds into the transmembrane crevice and participates in the orthosteric binding site for retinal (Palczewski et al., 2000). ECL2 also contributes to dopamine binding to the D2 receptor (Shi and Javitch, 2004). A chimeric study of the human-rat P2Y4 receptor revealed that ECL2 influenced agonist versus antagonist function (Herold et al., 2004) and point mutations in ECL2 produced constitutively active receptors (Klco et al., 2005) or inhibitory effects on signaling when ECL2 flexibility was affected (Avlani et al., 2007). In ECL2 of the α1B-AR, 11 of 16 mutants reduced NE potency, including nine that decreased NE affinity (W184A, K185A, E186A, N190A, D191A, D192A, C195A, T198A, and E200A) and four that reduced NE efficacy (N190A, C195A, G196A, and V197A). An earlier mutational study swapped three of these residues in ECL2 of α1B-AR to the corresponding residue in α1A-AR (G196Q, V197I, and T198N) and showed that these residues influence antagonist selectivity between these subtypes (Hwa et al., 1995; Zhao et al., 1996). Examining our model of α1B-AR, these three ECL2 residues are positioned where they might influence how NE reaches the orthosteric site in α1B-AR (see Figs. 6 and 7).

In our model of α1B-AR (Ragnarsson et al., 2013), ECL2 is stabilized by several intramolecular interactions in addition to being anchored to the extracellular end of TMH3 via a conserved cysteine bond that may promote stabilization of the inactive state (Massotte and Kieffer, 2005). Thus, it is not surprising that breaking the conserved disulfide bond bridging ECL2 to TMH3 in the C118A and C195A mutants had a dramatic effect on agonist potency, as shown previously for α1B-AR (Ragnarsson et al., 2013) and other class A GPCRs (Dixon et al., 1987; Karnik et al., 1988; Fraser, 1989; Dohlman et al., 1990; Karnik and Khorana, 1990; Kurtenbach et al., 1990; Noda et al., 1994; Perlman et al., 1995; Lin and Sakmar, 1996; Cook and Eidne, 1997; Zhou and Tai, 2000). The C118A and C195A mutants reduced both NE affinity and efficacy, indicating that this conserved disulfide bridge stabilizes a conformation that facilitates both NE access to its binding site and NE signaling. Mutations in TMH4 adjacent to ECL2 (P180A and L181A) and two residues adjacent to C195 (G196A and V197A) also reduced NE affinity and efficacy, supporting a scaffolding role for TMH4 that is critical for normal functioning of α1B-AR. Interestingly, the G183A mutant at the ECS of TMH4 had decreased prazosin and NE affinity, but unchanged efficacy, opposite to the effect seen for the G109A mutant in ECL1 (Ragnarsson et al., 2013).

In contrast to the majority of ECL1 and ECL2 mutations affecting α1B-AR function, only L323A in ECL3 decreased NE affinity, whereas the K324A and P326A mutants associated with TMH7 significantly reduced NE potency. A minor role played by ECL3 in receptor activation is in agreement with results from a study on the adenosine A1 receptor (Peeters et al., 2012), whereas ECL3 was shown to be more important for the β2-opioid receptor (Decaillot et al., 2003) and β2-AR activation (Zhao et al., 1998). Two mutations adjacent to ECL3 (F330A and K331A in TMH7) significantly enhanced NE affinity but reduced NE efficacy without affecting prazosin affinity (Ragnarsson et al., 2013).
In contrast, mutating the equivalent residue to F330 in the $\alpha_{1A}$-AR (Phe308) had no effect on antagonist binding and either showed no effect or decreased agonist affinity, depending on the agonist investigated (Waugh et al., 2001). A stabilizing salt bridge between D125 in TMH3 and K331 in TMH7 has been reported to maintain the inactive state of the receptor (Porter et al., 1996). Our $\alpha_{1B}$-AR model does not form this salt bridge, although both the F330A and K331A mutants had increased NE affinity and reduced NE efficacy, suggesting a complex effect on NE function that appears independent of a salt-bridge effect. Interestingly, a phenylalanine at a position equivalent to F330 in $\alpha_{1B}$-AR is conserved in all adrenoceptors, except $\beta_2$-AR (see Fig. 7).

The effects of ECS mutations on NE affinity and/or efficacy tended to cluster in different regions of the ECS (Fig. 6B). Mutations that reduced NE affinity alone clustered at the start of ECL1, across most of ECL2, and at the start of ECL3, whereas mutations that reduced efficacy alone appeared in small clusters in the central portion of ECL1 and the second half of ECL2. In contrast, mutations likely to have structural effects, including C118A, P180A, and C195A, had large effects on both affinity and efficacy, whereas mutations that enhanced NE affinity clustered at the TMH2-ECL1 and ECL3-TMH7 junctions, which are adjacent in GPCR structures. These results suggest that different regions of the ECS play discrete roles in GPCR function. Depending on position, mutating hydrophobic, polar, and charged residues to alanine all contributed to changed function. A functional heat map of the ECS (Fig. 7) revealed that residues lining the upper lip of the entrance to the NE binding pocket reduced NE affinity, whereas mutating residues lining the lower lip mostly enhanced NE affinity. Given that these residues line the entrance to the NE binding pocket, they likely

![Fig. 6. Structure of the ECS of the $\alpha_{1B}$-AR showing key residues involved in NE affinity and/or efficacy. (A) Top view of the $\alpha_{1B}$-AR ECS showing side chains for residues where mutations to alanine significantly changed NE $K_i$ (red), NE efficacy (blue), or both NE $K_i$ and efficacy (magenta) compared with WT $\alpha_{1B}$-AR. The backbone of ECS residues without effect is colored cyan. (B) Mutants that significantly changed NE $K_i$ (red), NE efficacy (blue), or both NE $K_i$ and efficacy (magenta) compared with WT $\alpha_{1B}$-AR, with $\uparrow$ 10-fold increased or $\downarrow$ decreased, $\downarrow\downarrow$ 10- to 100-fold decreased, or $\downarrow\downarrow\downarrow$ 100- to 1000-fold decreased responses indicated.](image)

![Fig. 7. Functional heat map of the ECS of the $\alpha_{1B}$-AR highlighting the position of mutations that influenced NE potency, affinity, and efficacy. Top view of the $\alpha_{1B}$-AR showing the backbone for mutated ECS residues that significantly affected (A) NE potency ($EC_{50}$), (C) NE affinity ($K_i$), or (E) NE efficacy compared with WT $\alpha_{1B}$-AR, orientated as shown in Fig. 1. Top view of the $\alpha_{1B}$-AR in the same orientation, showing the ECS with residues that significantly affected (B) NE potency ($EC_{50}$), (D) NE affinity ($K_i$), or (F) NE efficacy compared with WT $\alpha_{1B}$-AR. Mutations causing $\uparrow$ 10-fold increases (red) or decreases (light blue), 10- to 100-fold decreases (blue) or 100- to 1000-fold decreases (purple) are highlighted. Mutations at the ECS residues without effect are colored white, and non-ECS residues are shown in yellow.](image)
directly influence NE binding kinetics. In contrast, relatively few exposed residues greatly affected NE efficacy (mostly <10-fold), whereas buried or structurally significant residues tended to have more significant effects on NE efficacy.

Figure 8 shows sequence alignments for the ECS residues of hamster α1B-AR and selected class A GPCRs, including human α1B-AR, α1A-AR, α1D-AR, α2A-AR, α2C-AR, β1-AR, β2-AR, muscarinic M2 and M3 receptors, dopamine D3 receptor, histamine H1 receptor, and κ-opioid receptors. This alignment reveals that many of the residues in and adjoining ECL1 are conserved, whereas residues in ECL2 and ECL3 are less conserved. Interestingly, superimposing our mutational results on this alignment revealed that most of the functionally significant mutations are positioned in the more conserved regions of ECS, especially in the highly conserved ECL1 (Fig. 8). The broad potential for ECS mutations to affect GPCR function has relevance for the increasing number of nonsynonymous single-nucleotide polymorphisms (SNPs) now being identified in GPCRs through next-generation sequencing (Hecht et al., 2013). SNPs have the potential to alter receptor pharmacology and response to medication and can predispose people to various diseases (Thompson et al., 2008). SNPs of potential medical importance have been identified at the ECS of aminergic GPCRs (Kojima et al., 2009), including R166K in the human α1A-AR, which reduced both NE affinity and potency in 1B-AR, which also modified 1A-AR, the type of effect produced by the V329A mutation on 1B-AR function.

In conclusion, this study reveals that mutating ECS residues of GPCRs can have profound effects on agonist affinity and/or efficacy. At α1B-AR, the type of effect produced clustered to different regions of ECS, consistent with different regions of ECS playing discrete functional roles. Collectively, our data highlight that changes in NE potency result from effects on NE affinity, mostly through more conserved regions of ECL1 and ECL2 that surround the entrance to the NE binding pocket or are associated with the ECS of TMH4 and TMH7, or from effects on signaling efficacy that arise mostly through buried or structurally significant residues.

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