Role of the Second Extracellular Loop of the Adenosine A₁ Receptor on Allosteric Modulator Binding, Signaling, and Cooperativity


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ABSTRACT

Allosteric modulation of adenosine A1 receptors (A1ARs) offers a novel therapeutic approach for the treatment of numerous central and peripheral disorders; however, despite decades of research, there is a relative paucity of structural information regarding the A1AR allosteric site and mechanisms governing cooperativity with orthosteric ligands. We combined alanine-scanning mutagenesis of the A1AR second extracellular loop (ECL2) with radioligand binding and functional interaction assays to quantify effects on allosteric ligand affinity, cooperativity, and efficacy. Docking and molecular dynamics (MD) simulations were performed using an A1AR homology model based on an agonist-bound A2AAR structure. Substitution of E172ECL2 for alanine reduced the affinity of the allosteric modulators PD81723 and VCP171 for the unoccupied A1AR. Residues involved in cooperativity with the orthosteric agonist NECA were different in PD81723 and VCP171; positive cooperativity between PD81723 and NECA was reduced on alanine substitution of a number of ECL2 residues, including E170ECL2 and K173ECL2, whereas mutation of W146ECL2 and W156ECL2 decreased VCP171 cooperativity with NECA. Molecular modeling localized a likely allosteric pocket for both modulators to an extracellular vestibule that overlaps with a region used by orthosteric ligands as they transit into the canonical A1AR orthosteric site. MD simulations confirmed a key interaction between E172ECL2 and both modulators. Bound PD81723 is flanked by another residue, E170ECL2, which forms hydrogen bonds with adjacent K168ECL2 and K173ECL2. Collectively, our data suggest E172ECL2 is a key allosteric ligand-binding determinant, whereas hydrogen-bonding networks within the extracellular vestibule may facilitate the transmission of cooperativity between orthosteric and allosteric sites.

Introduction

G protein–coupled receptors (GPCRs) account for approximately 30% of the targets of prescription medicines (Overington et al., 2006). Most of these medicines act at the endogenous agonist (orthosteric) site; however, in many cases, orthosteric-based drug discovery remains suboptimal owing to a lack of sufficient selectivity between related GPCR subtypes (May et al., 2007b). One promising approach for achieving greater selectivity is to target topographically distinct allosteric sites on GPCRs (Christopoulos, 2002; May et al., 2007b; Wootten et al., 2013). Allosteric ligands have the potential to modulate the binding and/or signaling properties of an orthosteric ligand and/or modulate GPCR activity even in the absence of orthosteric ligand (May and Christopoulos, 2003; Christopoulos, 2014). Allosteric regions typically display greater sequence divergence across the GPCR subtypes, enabling greater subtype selectivity compared with the orthosteric domains. An additional advantage is that many allosteric modulators can selectively “tune” tissue responses either up or down when and where the endogenous agonist is present. This type of spatial and temporal specificity of action that can be achieved with allosteric modulators is unattainable with orthosteric ligands, which continuously stimulate or
inhibit receptor function where and when they are present (Christopoulos and Kenakin, 2002; May et al., 2007b).

The endogenous nucleoside adenosine acts via four adenosine GPCRs (ARs): A1AR, A2AAR, A2BAR, and A3AR (Fredholm et al., 2001). The highly subtype selective A1AR allosteric ligand, PD81723 (2-amino-4,5-dimethyl-3-thienyl)-(3-(trifluoromethyl)phenyl)methanone), was the first synthetic positive allosteric modulator (PAM) of GPCR agonist function identified (Fig. 1) (Bruns and Fergus, 1990; Bruns et al., 1990; May et al., 2010; Hill et al., 2014). The utility of A1AR PAMs has been demonstrated in the kidney (Park et al., 2012), heart (Bruns and Fergus, 1990; Valant et al., 2010), and neurons (Pan et al., 2001; Imlach et al., 2015), suggesting that A1AR PAMs provide a promising therapeutic avenue to treat renal and myocardial ischemia-reperfusion injury and chronic neuropathic pain. Given their therapeutic potential, numerous studies have investigated the structure-activity relationships of A1AR PAMs, predominantly exploring derivatives based on the 2-amino-3-benzothiophene scaffold (Romagnoli et al., 2015). Despite extensive derivation and interrogation of this core scaffold for more than two decades; however, existing A1ARs PAMs display largely low affinity, low cooperativity, and generally poor solubility.

Alternative approaches, such as structure-based studies, are thus required to assist the design of more potent, selective, and efficacious A1AR modulators. The recent high-resolution A2AAR crystal structures in both antagonist- and agonist-bound conformations have facilitated the generation of A1AR homology models (Jaakola et al., 2008; Dore et al., 2011; Lebon et al., 2011; Xu et al., 2011); however, there remains a relative paucity of information regarding the location of the A1AR allosteric site, reflecting the key challenges associated with structure-function analyses of allosterity, because the observed effect of any modulator in the presence of orthosteric ligand reflects a composite of at least three molecular properties: the affinity of the modulator for the unoccupied receptor, the cooperativity between the modulator and the orthosteric ligand when both are present, and the potential intrinsic signaling efficacy of each ligand; these properties can be governed by separate structure-activity and structure-function relationships (Christopoulos, 2014). Surprisingly, to date, only two structure-function studies have attempted to map the A1AR allosteric site, both of which implicated the second extracellular loop (ECL2) of the A1AR (specifically residues W156ECL2, E164ECL2, S150ECL2, and M162ECL2) in A1AR PAM activity (Peeters et al., 2012; Kennedy et al., 2014). Neither study, however, explicitly addressed which residues governed modulator affinity relative to transmission of cooperativity or direct allosteric agonism. Nonetheless, this work clearly highlighted ECL2 as important in the actions of the subtype selective 2-amino-3-benzothiophene A1AR allosteric modulators, as this region is the least conserved in both sequence and length across the AR subtypes.

In our accompanying article (Nguyen et al., 2016), we report our work demonstrating that ECL2 can significantly influence orthosteric agonist and antagonist pharmacology. Furthermore, ECL2 is involved in the “transition” of agonists into the orthosteric site within the transmembrane (TM) domain. In the present study, we assessed the influence of select A1AR-ECL2 alanine mutations on the affinity, cooperativity, and efficacy of the well characterized allosteric enhancer PD81723 and a more recent derivative, VCP171 ((2-amino-4-(3-(trifluoromethyl)phenyl)thiophen-3-yl)(phenyl)methanone) (Fig. 1) (Aurelio et al., 2009b). We have found that ECL2 contributes to a common allosteric pocket in the extracellular vestibule that overlaps with the transit pocket used by orthosteric ligands, identifies E172ECL2 as a vital residue for modulator affinity, and highlights how different residues can mediate cooperativity and direct agonism in a manner that can vary with the nature of the allosteric or orthosteric ligand under investigation.

### Materials and Methods

**Materials.** VCP171 was synthesized as described previously (Aurelio et al., 2009b; Valant et al., 2014); PD81723 was synthesized as described previously (Aurelio et al., 2009a). All other reagents were obtained from suppliers described in the accompanying article (Nguyen et al., 2016).

**Receptor Mutagenesis, Transfection, Cell Culture, and Receptor Expression.** Receptor mutagenesis, transfection, cell culture, and measurement of cell surface expression of wild-type (WT) and mutant human A1ARs containing a triple human influenza hemagglutinin (HA) N-terminal tag (3xHA-A1ARs) were performed as described in our accompanying article (Nguyen et al., 2016).

**Whole-Cell Radioligand Binding.** [3H]DPCPX (8-cyclopentyl-1,3-dipropylxanthine, [dipropyl-2,3-3H(N)]) whole-cell interaction binding assays on Chinese hamster ovary (CHO) Flp-In cells stably expressing the WT or mutant 3xHA-A1AR were performed at 4°C for 3 hours in a final volume of 100 μl of HEPES buffer (145 mM NaCl, 10 mM d-glucose, 5 mM KCl, 1 mM MgSO4, 10 mM HEPES, 1.3 mM CaCl2, 15 mM NaHCO3, pH 7.45) in the presence of 1 nM [3H]DPCPX, increasing concentrations of the orthosteric agonist 5’-N-ethylcarboxamidoadenosine (NECA) and/or allosteric ligand (PD81723 or VCP171). Nonspecific binding was defined with 1 μM of selective A1AR antagonist trans-4-[(2-phenyl-7H-pyrrolo[2,3-d]pyrimidin-4-yl)aminocyclohexanol. Assays were then terminated by washing twice with 100 μl of cold phosphate-buffered saline (PBS) per well, followed by the addition of 100 μl of OptiPhase Supermix scintillation cocktail and bound radioactivity was measured using a MicroBeta2 plate counter (PerkinElmer, Waltham, MA).

**Inhibition of cAMP Accumulation.** Inhibition of cAMP accumulation was assessed as described previously (Baltos et al., 2016). Briefly, Flp-In-CHO cells stably expressing the WT or mutant 3xHA-A1AR were seeded at 20,000 cells/well in a 96-well plate and incubated at 37°C overnight. Interaction assays were performed at 37°C in stimulation buffer (140 mM NaCl, 5.4 mM KCl, 0.8 μM MgSO4, 0.2 mM Na2HPO4, 0.44 mM KH2PO4, 1.3 mM CaCl2, 5.6 mM d-glucose, 0.5 mM HEPES, 0.1% bovine serum albumin, and 10 μM rolipram, pH 7.45). Cells were washed and incubated in stimulation buffer for 30 minutes, followed by a 10-minute exposure to allosteric modulator alone and then the addition of 3 μM forskolin in the absence and presence of increasing concentrations of NECA. After a 30-minute incubation at 37°C, the reaction was terminated by rapid removal of
buffer and the addition of 50 µl of ice-cold ethanol. After ethanol evaporation, detection was performed as outlined previously (Baltos et al., 2016; Vecchio et al., 2016).

**Data Analysis.** Data were analyzed using nonlinear regression analysis software (GraphPad Prism 6.0, La Jolla, CA). Equilibrium binding-interaction experiments were fitted to the allosteric ternary complex model in eq. (1) (Leach et al., 2010):

\[
Y = \frac{B_{\text{max}}[A]}{[A]^n + \left(\frac{K_A K_B}{K_A + K_B}\right) \left(1 + [A] K_A + [B] K_B + \alpha [A] [B] K_A K_B\right)}
\]

where \(Y\) is specific binding; \(B_{\text{max}}\) is the relative receptor expression; and \(K_A, K_B, \) and \(K_t\) represent the equilibrium dissociation constants of \([^3\text{H}]\)DPCPX (A), the allosteric ligand (B), and NECA (I), respectively. The binding cooperativity between the allosteric ligand and \([^3\text{H}]\)DPCPX or NECA is denoted by \(\alpha_A\) or \(\alpha_I\), respectively. A cooperativity factor \(\alpha > 1\) describes the positive cooperativity; a value \(0 < \alpha < 1\) describes negative cooperativity, and a value of \(\alpha = 1\) describes neutral cooperativity.

Concentration-response curves for the interaction between NECA and allosteric ligands PD81723 and VCP171 in a cAMP accumulation assay were globally fitted to the operational model of allosterism in eq. (2) (Leach et al., 2007):

\[
\]

where \(E_m\) is the maximal cellular response, and \(K_A\) and \(K_B\) are the equilibrium dissociation constants of NECA (A) and the allosteric ligand (B), respectively; \(\tau_A\) and \(\tau_B\) are operational measures of NECA and allosteric ligand efficacy, respectively; \(\alpha\) is the binding cooperativity factor; and \(\beta\) denotes the magnitude of the allosteric effect on orthosteric agonist efficacy; and \(n\) is the slope of the transducer function that links occupancy to response. Orthosteric agonist and allosteric ligand affinity were constrained to values determined from radioligand binding (Table 1 and accompanying article). Efficacy parameters (\(\tau_A\) and \(\tau_B\)) were corrected for differences in receptor expression between mutants by using the \(B_{\text{max}}\) values determined from saturation binding assays to scale the efficacy (\(\tau\)) parameters to those of the WT receptor (Gregory et al., 2010). All data preferentially were fitted to a transducer slope (\(n\)) equal to 1, as determined by an extra-sum-of-squares test (F test).

All values of potency, affinity, efficacy, and cooperativity were estimated as logarithms (Christopoulos, 1998). Statistical analysis was performed using a one-way analysis of variance with a Dunnett’s post hoc test to determine differences between the WT and mutant 3xHA-A1AR mutants. Statistical significance was defined as \(P < 0.05\).

**Homology Modeling, Docking, and Molecular Dynamics Simulations.** The generation of the active-like homology model of the A1AR based on the agonist-bound human A2AAR (PDB ID 3QAK) was described in our accompanying article (Nguyen et al., 2016). The ICM Pocket Finder (Molsoft, LLC, San Diego, CA) algorithm was used to predict potential ligand binding sites, and NECA, PD81723, and VCP171 were docked using ICM version 3.8.0 (with default parameters) (An et al., 2005, Abagyan and Kufareva, 2009). The top scoring docking conformation for each ligand was selected and prepared for molecular dynamics (MD) simulations. MD simulations of the final complex were carried out with the NAMD 2.10 (Phillips et al., 2005) package using the three-site rigid water TIP3P model, CHARMM27 (MacKerell et al., 1998; Mackrell et al., 2004), and CGenFF (Vanommeslaeghe et al., 2010; Yu et al., 2012) v3.0.1 force fields as described previously (Shonberg et al., 2013) (http://www.ks.uiuc.edu/Training/Tutorials/science/membrane/mem-tutorial.pdf). The particle mesh Ewald (Essmann et al., 1995) method was used to evaluate

### Table 1

The A1AR homology model and associated MD simulations were used to generate allosteric site candidates and to study allosteric interactions. All values of potency, affinity, efficacy, and cooperativity were estimated as logarithms (Christopoulos, 1998). Statistical analysis was performed using a one-way analysis of variance with a Dunnett’s post hoc test to determine differences between the WT and mutant 3xHA-A1AR mutants. Statistical significance was defined as \(P < 0.05\).
electrostatic interactions. Each system contained an A1AR, NECA, and either PD81723 or VCP171 in the presence of a lipid bilayer composed of ∼220 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine molecules generated using the membrane plugin of the VMD software (v. 1.9.2) (Humphrey et al., 1996), and ∼15,800 water molecules. Sodium and chloride ions were added to neutralize the system, with extra NaCl added to reach a final concentration of 150 mM (∼35 sodium ions, ∼48 chloride ions). MD energy minimization, equilibration, and the production run were performed as described within the accompanying article. VMD v. 1.9.2 was used for the visualization and analysis of the residue-ligand contacts through the course of each simulation using inhouse scripts.

Results

Expression of Human A1AR ECL2 Alanine Mutants. As reported in our accompanying article that investigated the role of ECL2 on orthosteric ligand pharmacology (Nguyen et al., 2016), each residue within ECL2 was substituted for alanine, with the exception of C169ECL2, which is known to form an important disulfide bond with C803.35 in TM3. Superscripts refer to Ballesteros-Weinstein residue numbering (Ballesteros and Weinstein, 1995). Alanine substitutions were made in the 3xHA-A1AR, which is a human A1AR-containing a triple HA N-terminal tag. The pharmacology of the allosteric ligands PD81723 and VCP171 was equivalent at the 3xHA-A1AR and untagged human A1AR (data not shown).

In all cases, single alanine mutations were introduced, with the exception of S150ECL2A + V151ECL2A (SV150ECL2AA) and M162ECL2A + G163ECL2A + E163ECL2A (MGE162ECL2AAA), for which double and triple mutations were substituted and well expressed. Furthermore, as outlined in our accompanying article (Nguyen et al., 2016), although the 3xHA-A1AR containing the alanine substitution of F171ECL2 was expressed to similar levels as the WT (Supplemental Fig. 1 of a accompanying article), orthosteric ligand binding and function could not be detected; therefore, this mutation was not investigated in the current study.

Influence of A1AR-ECL2 Alanine Substitutions on Allosteric Ligand Affinity and Cooperativity. Whole-cell [3H]DPCPX interaction binding assays in the absence and presence of increasing concentrations of the orthosteric agonist NECA, and allosteric ligands were performed at WT and mutant 3xHA-A1AR (Fig. 2, A and B). Fitting of these data to an allosteric ternary complex model allowed estimation of allosteric ligand affinity (pK_B) and binding cooperativity (log(α_A)) between the allosteric ligand and orthosteric agonist (Table 1); in most cases, the cooperativity between the modulators and [3H]DPCPX was highly negative, and thus the value of log(α_A) was constrained to a very low value of −3 (i.e., α_A = 0.001), making it indistinguishable from competitive inhibition. At the WT 3xHA-A1AR, PD81723 and VCP171 had micromolar affinity for the allosteric site on the unoccupied receptor and enhanced the affinity of NECA approximately 4- to 8-fold, depending on the PAM. The only mutation that resulted in a significant decrease in the affinity of PD81723 and VCP171 for the free (unoccupied) receptor was E172ECL2A (Fig. 3). Indeed, E172ECL2A was the only ECL2 mutation to significantly influence VCP171 affinity, albeit to a lesser extent than PD81723 (Fig. 3B). In contrast, an increase in the affinity of PD81723 was observed upon alanine substitution of nine residues: N147ECL2, N148ECL2, S161ECL2, V166ECL2, I167ECL2, K168ECL2, K173ECL2, and N175ECL2 (Fig. 3A).

The ECL2 mutations that significantly influenced allosteric binding cooperativity with the orthosteric agonist NECA were probe-dependent, that is, not conserved between PD81723 and VCP171. With respect to PD81723, three pairs of mutations significantly decreased PD81723 binding cooperativity with the agonist: N147ECL2A and N148ECL2A, G160ECL2A and S161ECL2A, E170ECL2A and K173ECL2A (Fig. 3A). With the exception of G160ECL2, each of these mutations was also associated with a concurrent increase in PD81723 affinity. With respect to VCP171, alanine substitution of two tryptophan residues, W146ECL2 and W156ECL2, caused a significant decrease in the binding cooperativity between VCP171 and NECA, with no significant effect on VCP171 affinity (Fig. 3B). For comparison, the composite parameter, log(α_A) + pK_B, which reflects the affinity of each modulator on the NECA-occupied receptor, is also shown in Table 1, where it can be seen that far more subtle effects of each mutation would be concluded if the individual contributions of amino acids to the affinity for the unoccupied receptor relative to cooperativity were not evaluated.

Influence of ECL2 Alanine Substitutions on Allosteric Ligand Efficacy and Functional Cooperativity with NECA. Functional interaction assays in the absence and presence of increasing concentrations of orthosteric agonist and/or allosteric ligands were performed at WT and mutant 3xHA-A1AR (Fig. 2, C and D). Data were fitted to an operational model of allostery to estimate allosteric ligand efficacy corrected for changes in receptor expression (log(β(CD))) and functional cooperativity (log(β(cd))) between the allosteric ligand and the orthosteric agonist NECA (Table 2). Functional interaction assays performed in the absence and presence of adenosine deaminase were comparable, suggesting minimal influence of endogenous adenosine (unpublished results). Similar to previous findings for Flp-In-CHO cells stably expressing...
the WT A1AR (Vecchio et al., 2016), no evidence for constitutive activity was apparent for WT or mutant A1ARs (unpublished results).

In the absence of orthosteric agonist, both PD81723 and VCP171 behaved as allosteric partial agonists at the WT 3xHA-A1AR, mediating an increase in the inhibition of forskolin-stimulated cAMP accumulation (Fig. 2, C and D).

Analysis of interaction experiments between increasing concentrations of each modulator and NECA found that residues involved in the transmission of direct allosteric ligand efficacy ($\tau_{B(C)}$) were relatively conserved between PD81723 and VCP171. A significant decrease in the efficacy of PD81723 and VCP171 was observed at N148ECL2A, E153ECL2A, S161ECL2A, and I167ECL2A (Fig. 3). A 5- to 10-fold decrease

### Table 2

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<th>log$_{10}$B(C)</th>
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*Logarithm of the efficacy parameter determined from the operational model of allosterism corrected for changes in receptor expression.

Logarithm of the functional cooperativity between the allosteric ligand and NECA. Antilogarithm is shown in parentheses.

*P < 0.05, significantly different from WT, one-way analysis of variance, Dunnett’s post hoc test.
in efficacy of PD81723 and VCP171 was also observed at R154ECL2A and I175ECL2A; however, this reached significance only for VCP171 (Fig. 3). Both PAMs enhanced NECA potency at the WT 3xHA-A1AR approximately 4-fold. Interestingly, whereas E172ECL2A caused a small increase in the functional cooperativity ($\log(\alpha)$) between PD81723 and the agonist (Fig. 3A), the remaining mutations had no significant effect. For VCP171, two clusters of ECL2 mutations caused a small but significant decrease in VCP171 functional cooperativity. The first cluster involved R154ECL2A, W156ECL2A, and N159ECL2A, and the second involved V174ECL2A and I175ECL2A (Fig. 3B). These results may suggest a divergence in the influence of the mutations on cooperativity with NECA at the level of binding affinity ($\alpha$) versus efficacy ($\beta$). However, evaluation of $\log(\beta)$ values found no significant difference from the WT, with the exception of the influence of S161ECL2A on PD81723, suggesting that the observed cooperativity between NECA and the modulators is driven largely by changes in binding cooperativity ($\alpha$) (Supplemental Fig. 1A).

**Molecular Modeling of the A1AR Allosteric Site.** As described in the accompanying article (Nguyen et al., 2016), an “active-like” homology model of the human A1AR was generated based on the agonist (UK432097)-bound human A2AAR crystal structure. The ICM PocketFinder software identified three potential binding pockets within this model. One pocket was located within the TM bundle and corresponded to the well-characterized deep orthosteric site, whereas the other two pockets were located within different regions of the extracellular vestibule. NECA was docked into the deep orthosteric pocket in the TM region, and the allosteric modulators were docked into the putative allosteric sites within the extracellular vestibule. MD simulations were performed for each A1AR homology model cobound with the NECA and either PAM. When PD81723 or VCP171 were docked into the pocket located exclusively within ECL2, both PAMs dissociated from the binding site in four of the five 10 ns MD simulations. Furthermore, this binding pocket does not involve E172ECL2, the only ECL2 residue that significantly decreased A1AR allosteric ligand affinity when mutated to alanine. Collectively, these results suggest that this pocket is unlikely to represent the A1AR allosteric binding site.

The second putative allosteric pocket was located within the extracellular vestibule bordered by TM2, ECL2, TM6, ECL3, and TM7 (red in Fig. 4). PD81723 and VCP171 were more stable in this pocket, remaining bound during the entire 40 ns MD simulation in all but one of the seven MD simulations (Fig. 5). Residues located within 3.5 Å of PD81723 for an average 60%–70% of the 40 ns run time for each of the six MD simulations were F171ECL2, E172ECL2, and N254ECL2 (Fig. 6A). Within the extracellular vestibule, PD81723 was predicted to form hydrogen bonds with E172ECL2, with the highest frequency compared with the other residues (Fig. 7; Supplemental Material 1), which correlates well with the significant reduction in PD81723 affinity observed for the E172ECL2A mutation (Fig. 7C). PD81723 was also flanked by a hydrogen bond with the side chain of another glutamic acid residue, E170ECL2 (Fig. 7B). This residue was found to reside within 3.5 Å of PD81723 for greater than 20% of total MD time in two of the six simulations (Fig. 6A). Our molecular modeling predicted a network of hydrogen bond interactions between residues within the extracellular vestibule surrounding the allosteric binding pocket (Fig. 7B). These include a hydrogen bond between E172ECL2 and K265ECL3 as well as a hydrogen bond between the side chain of E170ECL2 and two lysine residues, K168ECL2 and K173ECL2 (Fig. 7B). Our mutagenesis results suggest that both E170ECL2 and K173ECL2 are involved in the transmission of PD81723 binding cooperativity (Fig. 7D). As such, in addition to stabilizing the A1AR extracellular structure, the putative network of hydrogen bond interactions within the extracellular vestibule may have a role in the transmission of cooperativity between the extracellular and orthosteric binding sites.
ECL2 residues located within 3.5 Å of VCP171 during the 40 ns MD simulations include F171ECL2 and E172ECL2 (Fig. 6B). Similar to PD81723, VCP171 was stabilized in the allosteric pocket via a hydrogen bond with E172ECL2, the only residue for which a significant reduction in VCP171 affinity was observed upon mutation to alanine (Fig. 8; Supplemental Material 2). Collectively, these MD simulations support our experimental finding that E172ECL2 is a critical determinant for allosteric ligand binding.

Discussion

The A1AR is a potential therapeutic target for a number of conditions. A1AR allosteric modulators offer considerable advantages over their orthosteric counterparts; however, discovery efforts have yet to yield A1AR-selective allosteric modulators with high affinity and/or substantially diverse chemotypes. Medicinal chemistry efforts can be enriched by efforts that allow localization of the A1AR allosteric site coupled with delineation of structural determinants that govern affinity, cooperativity, and efficacy of allosteric ligands. Numerous class A GPCR allosteric sites recognized by exogenous ligands are thought to reside within the extracellular vestibule (Voigtländer et al., 2003; Avlani et al., 2007; Jäger et al., 2007; Narlawar et al., 2010; Nawaratne et al., 2010; Peeters et al., 2012; Abdul-Ridha et al., 2014). As such, the aim of the current study was to delineate the role of ECL2 in A1AR allosteric ligand pharmacology. We performed an alanine scan of ECL2 and quantified the effect of mutations on allosteric ligand affinity, cooperativity, and efficacy of allosteric ligands. To date, only a few studies have attempted to map the A1AR allosteric site. Two recent structure-function studies implicated ECL2 (Peeters et al., 2012; Kennedy et al., 2014); however, neither study differentiated residues involved in allosteric binding affinity versus cooperativity or efficacy. Peeters et al. (2012) suggested alanine substitution of W156ECL2 decreased PD81723 allosteric activity. Our results suggest that the mechanism behind this effect is because W156ECL2 is one of two tryptophan residues involved in mediating binding cooperativity between A1AR PAMs and orthosteric agonists, in our case specifically between VCP171 and NECA (Fig. 8, D and F). The recent high-resolution crystal structure of an active human M2 muscarinic acetylcholine receptor bound to both an orthosteric agonist and PAM identified extensive π-π stacking interactions between the aromatic ring of the PAM and aromatic residues in ECL2 and the top of TM7 (Kruse et al., 2013). Similar π-π stacking interactions may occur between the aromatic rings of VCP171 and the tryptophan residues W156ECL2 and W146ECL2 in the A1AR-ECL2; however, long time-scale MD simulations may be required to observe such interactions. A second study by Kennedy et al. (2014) combined alanine scanning and radioligand dissociation kinetic assays with molecular modeling and observed a significant decrease in A1AR PAM, ATL525 (2-amino-4,5,6,7-tetrahydro-benzo[b]thiophen-3-yl) biphenyl-4-yl-methanone), activity at two constructs containing alanine mutations within ECL2: N147ECL2, N148ECL2, G160ECL2, and N159G160S161M162. In agreement with these results, we observed a significant decrease in the binding cooperativity between PD81723 and NECA upon single alanine substitution of N147ECL2, N148ECL2, G160ECL2, or S161ECL2. In contrast to our results, Kennedy et al. (2014) identified S150ECL2 and M162ECL2 as key residues involved in conferring allosteric activity of ATL525; however, we have shown that the residues involved in the transmission of allosteric cooperativity appear to be probe-dependent. This probe dependence may be due to actual...
differences in binding modes/poses or, as suggested by our current work, due to differences in the transmission of cooperativity from a “common” allosteric binding domain.

The combination of our mutational and computational data suggests that E172ECL2 is a key residue involved in binding of both PD81723 and VCP171 to the nonagonist-occupied A1AR. The putative allosteric site was bordered by E172ECL2. The 2-amino and 3-keto group of PD81723 and VCP171 were predicted to form hydrogen bond interactions with E172ECL2 during MD simulations of cobound orthosteric and allosteric ligands; both the 2-amino and 3-keto groups are known to be required for A1AR allosteric ligand activity (Bruns et al., 1990; Kennedy et al., 2014). Of particular note, this proposed allosteric binding pocket, supported by our experimental findings and MD, overlaps with the “transition site” within the extracellular vestibule proposed for the orthosteric agonist NECA and partially overlaps with the binding site of the orthosteric antagonist DPCPX in our accompanying article (Nguyen et al., 2016). This finding may explain prior studies where relatively subtle structural changes in A1AR molecules can convert allosteric modulators into compounds with apparently orthosteric pharmacology (i.e., these changes can alter the thermodynamic properties that favor recognition of one pocket over the other) (Aurelio et al., 2010, 2011; Valant et al., 2012). Furthermore, the predicted partial overlap between the putative allosteric pocket and DPCPX provides a potential mechanism to explain the previously proposed competitive interaction between A1AR allosteric modulators and orthosteric antagonists (Bruns et al., 1990), as well as our findings of an inhibitory interaction between either PD81723 or VCP171 with [3H]DPCPX.

Prior studies of the M2 muscarinic acetylcholine receptor also identified a role for charged residues within ECL2 in the affinity and/or cooperativity of allosteric ligands (Gnagey et al., 1999; May et al., 2007a). In our study, alanine substitution of the charged A1AR ECL2 residues E170ECL2 and K173ECL2 significantly decreased the positive cooperativity between the NECA and PD81723. Furthermore, alanine substitution of E172ECL2 significantly decreased allosteric ligand affinity. Interestingly, at both the A1AR and M2 receptor, the cluster of charged residues implicated in allosteric ligand binding and/or the transmission of cooperativity is proximal to the conserved cysteine residue within ECL2 that forms a disulfide bond with TM3. Furthermore, our molecular modeling suggests multiple negatively charged glutamic acid and positively charged lysine residues located within the extracellular vestibule are involved in a network of hydrogen bonds. During MD simulations, the side chain of E170ECL2 forms a hydrogen bond with PD81723 and the side chain of two
adjacent lysine residues, K168ECL2 and K173ECL2. Further, E172ECL2 also makes a hydrogen bond with K265ECL3, shaping the extracellular region of the A1AR. These findings further highlight the potential role of charged residues within the extracellular vestibule in allosteric ligand actions at the A1AR.

The positive binding cooperativity for PD81723 was substantially reduced upon alanine substitution of N147ECL2, N148ECL2, G160ECL2, and S161ECL2. Glycine is the smallest amino acid owing to its lack of a side chain. Consequently, this amino acid has the greatest conformational flexibility, a mobility that may be required for the transmission of cooperativity. The polar asparagine and serine residues implicated in the transmission of cooperativity are located relatively far from the predicted allosteric site and therefore likely have indirect effects. Alternatively, the middle section of ECL2 may form a lid over the allosteric binding site and stabilize the conformation of the allosteric pocket; however, such large-scale movements are unlikely to be observed during the 40 ns MD simulations.

Nonetheless, the use of analytical approaches that differentiate mutational effects on modulator affinity from those that govern allosteric efficacy or that mediate the transmission of cooperativity with orthosteric ligands raises at least two key issues for future consideration, both related to the role of the transition of the unliganded receptor to a bound active state. The first issue is exemplified by the decrease in signaling efficacy of both PD81723 and VCP171 upon alanine substitution of N148ECL2, E153ECL2, R154ECL2, S161ECL2, I167ECL2, or I175ECL2. In our accompanying article (Nguyen et al., 2016), a significant decrease in the efficacy of the orthosteric agonist, NECA, was also reported at the N148ECL2A, E153ECL2A, and R154ECL2A mutations, suggesting that these residues are part of a “global” activation mechanism irrespective of the “trigger” (i.e., allosteric or orthosteric). In contrast, S161ECL2, I167ECL2, and I175ECL2 appear to be exclusively involved in the transmission of allosteric but not orthosteric ligand efficacy. The paradigm of global versus allosteric modulator-specific activation mechanisms has been reported for other receptors (e.g., see Nawaratne et al., 2010). The second key issue relates to mechanisms underlying the transmission of cooperativity. Although our mutational analysis of modulator affinity was determined explicitly within the context of the free receptor, changes in cooperativity reflect effects on the ternary complex of agonist-receptor-modulator. It is likely that the nature of the allosteric pocket changes dramatically between the two states; this would not be captured by the ground-state K_B value but would be subsumed in the cooperativity values. Thus, the hydrogen bond networks implicated as playing a

Fig. 8. Predicted binding mode of VCP171 at the partially active A1AR homology model. (A) Side view of the A1AR homology model (light blue) cobound with NECA (green sticks) and VCP171 (yellow carbon sticks). (B) Key residue (green sticks) bordering the VCP171 (yellow carbon sticks) binding site that were involved in hydrogen bond interactions (black dotted line) during the MD simulations. (C–F) Residues at which alanine substitutions significantly decreased (orange sticks) VCP171 affinity (C), binding cooperativity (D), efficacy (E), and functional cooperativity (F). Figures were generated using PYMOL.
role in the transmission of allostery may be part of a larger, more dynamic network that reflects receptor transition between states and to which ECL2 contributes an important role. This can also explain why some of the residues that have a significant effect on ligand pharmacology, particularly signaling efficacy or cooperativity, need not be in the immediate vicinity of any predicted binding pocket.

In conclusion, the current study has provided new insights into the location of the A1AR allosteric site, as well as residues involved in the transmission of allosteric cooperativity and efficacy. Residues involved in allosteric ligand binding and efficacy were conserved between the two allosteric ligands assessed, whereas residues involved in the transmission of allosteric cooperativity were distinct. The delineation of ECL2 residues involved in allosteric ligand affinity, cooperativity, and efficacy provided within this study may facilitate future structure-function studies of the A1AR allosteric site and assist structure-based design of novel A1AR allosteric ligands.

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Authorship Contributions

Participated in research design: A.T.N. Nguyen, Sexton, May, Christopoulos.
Conducted experiments: A.T.N. Nguyen, Vecchio, Aurelio.
Wrote or contributed to the writing of the manuscript: A.T.N. Nguyen, Scammells, White, Sexton, Gregory, May, Christopoulos.

References


Address correspondence to: Arthur Christopoulos and Lauren T. May, Drug Discovery Biology and Department of Pharmacology, Monash Institute of Pharmaceutical Sciences, Monash University, 399 Royal Parade, Parkville, VIC 3052, Australia. E-mail: arthur.christopoulos@monash.edu, lauren.may@monash.edu