Extracellular loop 2 of the adenosine A1 receptor has a key role in orthosteric ligand affinity and agonist efficacy


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Corresponding Author

To whom correspondence should be addressed:

Lauren May and Arthur Christopoulos

Drug Discovery Biology and Department of Pharmacology,

Monash Institute of Pharmaceutical Sciences,

Monash University

399 Royal Parade, Parkville,

VIC 3052, Australia

Tel.: (613) 9903-9095

Fax: (613) 9903-9581

E-mail: lauren.may@monash.edu, arthur.christopoulos@monash.edu

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3xHA-A₁AR, human A₁AR containing an amino terminal triple human influenza hemagglutinin epitope tag; [³H]DPCPX, 8-cyclopentyl-1,3-dipropylxanthine [dipropyl-2,3-³H(N)]; A₁AR, adenosine A₁ receptor; AR, adenosine receptor; CHO, Chinese hamster ovary; DMEM, Dulbecco’s Modified Eagle Medium; DOPE, Discrete Optimized Protein Energy; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; ECL2, second extracellular loop; FBS, fetal bovine serum; GPCR, G protein-coupled receptor; HA, human influenza hemagglutinin; IRI, ischemia reperfusion injury; MD, molecular dynamics; NECA, 5'-N-ethylcarboxamidoadenosine; PBS, phosphate buffered saline; PME, particle mesh Ewald; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; SLV 320, trans-4-[(2-Phenyl-7H-pyrrolo[2,3-d]pyrimidin-4-yl)amino]cyclohexanol; TM, transmembrane; UK432097, 6-[2,2-di(phenyl)ethylamino]-9-[(2R,3R,4S,5S)-5-(ethylcarbamoyl)-3,4-dihydroxyoxolan-2-yl]-N-[2-[(1-pyridin-2-ylpiperidin-4-yl)carbamoylamino]ethyl]purine-2-carboxamide; WT, wild-type; ZM241385, 4-(2-[7-amino-2-(2-furyl)-[1,2,4]triazolo- [2,3-a][1,3,5]triazin-5-ylamino]ethyl)-phenol.
Abstract

The adenosine A₁ G protein-coupled receptor (A₁AR) is an important therapeutic target implicated in a wide range of cardiovascular and neuronal disorders. Although it is well established that the A₁AR orthosteric site is located within the receptor’s transmembrane (TM) bundle, prior studies have implicated extracellular loop 2 (ECL2) as having a significant role in contributing to orthosteric ligand affinity and signaling for various G protein-coupled receptors (GPCRs). We thus performed extensive alanine scanning mutagenesis of the A₁AR-ECL2 to explore the role of this domain on A₁AR orthosteric ligand pharmacology. Using quantitative analytical approaches and molecular modeling, we identify ECL2 residues that interact either directly or indirectly with orthosteric agonists and antagonists. Discrete mutations proximal to a conserved ECL2-TM3 disulfide bond selectively impacted orthosteric ligand affinity, whereas a cluster of five residues near the TM4-ECL2 juncture influenced orthosteric agonist efficacy. A combination of ligand docking, molecular dynamics simulations and mutagenesis results suggest the orthosteric agonist, 5′-N-ethylcarboxamidoadenosine (NECA), binds transiently to an extracellular vestibule formed by ECL2 and the top of TMs 5 and 7, prior to entry into the canonical TM bundle orthosteric site. Collectively, this study highlights a key role for ECL2 in A₁AR orthosteric ligand binding and receptor activation.
Introduction

The adenosine receptor (AR) family of G protein-coupled receptors (GPCRs) consists of four subtypes, the A₁AR, A₂AAR, A₂BAR, and A₃AR. The A₁AR is widely distributed within the body with high levels found in the central nervous system and peripheral organs (Palmer and Stiles, 1995; Fredholm et al., 2001; Yaar et al., 2004). Preferentially coupled to Gᵢ/o proteins, A₁AR stimulation can reduce cardiac and renal ischemia reperfusion injury, atrial fibrillation and neuropathic pain (Fredholm et al., 2001). Further, A₁AR antagonists may be beneficial as potassium-saving diuretics (Jacobson and Gao, 2006; Müller and Jacobson, 2011) and cognition enhancers (Hess, 2001). Consequently, the A₁AR is an attractive therapeutic target and a detailed understanding of ligand binding and function is imperative for this clinically relevant target.

Recently, structures of the human A₂AAR co-crystallized with various agonists and antagonists have been solved, providing deep insight into the orthosteric ligand binding region (Jaakola et al., 2008; Lebon et al., 2011; Xu et al., 2011; Doré et al., 2011; Congreve et al., 2012; Hino et al., 2012; Liu et al., 2012; Lebon et al., 2015). These crystal structures suggest that both helical transmembrane (TM) domains and extracellular loops (ECLs) contribute to the geography of the orthosteric pocket. In particular, an integral role for the helical region of ECL2 was highlighted, with Phe168 and Glu169 in this loop forming a π-stacking interaction and hydrogen bonding, respectively, with both antagonist and agonists.

Previous homology modeling and mutagenesis studies suggested that the A₁AR orthosteric binding pocket is located within the TM bundle, with key roles attributed to residues L88³.³³, T91³.³⁶, Q92³.³⁷ and T277⁷.⁴² (Townsend-Nicholson and Schofield, 1994; Rivkees et al., 1999). However ECL2, a region with high sequence variability across the adenosine receptor subtypes, has also been implicated in A₁AR orthosteric agonist and antagonist pharmacology (Olah et al., 1994). Similarly, an emerging picture of the contribution of ECL2 in orthosteric ligand recognition, and even the
ability of GPCRs to transition between multiple functional states, has been more broadly suggested for other AR subtypes and family A GPCRs, including the A2AAR, A3AR, cannabinoid receptor 1, M2 and M3 muscarinic acetylcholine receptors, rhodopsin, β-adrenoceptors, dopamine receptors, chemokine receptor 4 and V1a vasopressin receptor (Olah et al., 1994; Kim et al., 1996; Howl and Wheatley, 1996; Gao et al., 2002; Shi and Javitch, 2004; Avlani et al., 2007; Scarselli et al., 2007; Gregory et al., 2010; 2012; Sabbadin et al., 2015). In particular, ECL2 residues in the area adjacent to the highly conserved disulfide-bonding cysteine appear to play a key role in agonist binding and function at family A GPCRs (Wheatley et al., 2012). At the human A2AAR, glutamate residues in ECL2 have also been suggested to be involved in orthosteric ligand recognition (Kim et al., 1996). Furthermore, a recent seminal study using unbiased long time-scale molecular dynamics (MD) simulations highlighted a role for ECL2 in entry and egress of β-adrenoceptor orthosteric ligands from the binding pocket. Interestingly, in that study, the trajectory of ligands into the orthosteric pocket involved a metastable state in an extracellular vestibule, delineated by ECL2 and ECL3, prior to entry into the binding pocket within the helical bundle (Dror et al., 2011). It is possible that this paradigm extends to other receptors, and that the residence time a ligand spends in this metastable state prior to engaging the canonical orthosteric site may have implications for receptor activation dynamics and potential allosteric targeting.

Therefore, the aim of the current study was to establish the role of the A1AR-ECL2 in orthosteric ligand binding and function using a combination of mutagenesis, quantitative analytical pharmacology and molecular modeling. Complete alanine scanning of ECL2 residues was performed and the effects on orthosteric ligand affinity and efficacy quantified using radioligand binding and cAMP accumulation assays. Interpretation of mutagenesis data was facilitated by ligand docking into A1AR homology models based on agonist and antagonist bound A2AAR crystal structures followed by MD simulations. Ligand docking poses were evaluated by identification and mutational validation of novel ligand-receptor interactions within the A1AR. Collectively, our
results demonstrate a key role for ECL2 in orthosteric ligand binding and A$_1$AR activation. We propose that ECL2, together with the top of TM5 and 7, forms an extracellular vestibule that is recognized by NECA (5'-N-ethylcarboxamidoadenosine) and facilitates the transition of this agonist into the orthosteric pocket within the transmembrane bundle. This vestibule may also represent a target for recognition of the A$_1$AR by allosteric ligands.
Materials and Methods

Materials

Chinese hamster ovary (CHO) Flp-In<sup>TM</sup> cells, Dulbecco’s modified Eagle’s medium (DMEM) and hygromycin B (Hygrogold<sup>TM</sup>) were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was purchased from ThermoTrace (Melbourne, VIC, Australia). The Quickchange II<sup>TM</sup> site-directed mutagenesis kit was purchased Agilent (La Jolla, CA). AlphaScreen<sup>TM</sup> reagents, OptiPhase Supermix<sup>TM</sup> scintillation cocktail and [3H]DPCPX (8-cyclopentyl-1,3-dipropylxanthine, [dipropyl-2,3-<sup>3</sup>H(N)]; specific activity, 120 Ci/mmol) were obtained from PerkinElmer Life Sciences. Primers were purchased from GeneWorks (Hindmarsh, SA, Australia). All other reagents were purchased from Sigma-Aldrich or Tocris and were of analytical grade.

Receptor Mutagenesis

Mutations were introduced into the coding sequence of the human A<sub>1</sub>AR containing an amino terminal triple human influenza hemagglutinin (HA) epitope tag (3xHA-A<sub>1</sub>AR) in pcDNA3.1+ vector from Missouri S&T cDNA Resource Center (Rolla, MO). Sequence was amplified by PCR using the following primers 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGCCACCACTTAAGCTTGGTACCACCTG-3′ (N-terminal forward primer including attB, Kozak sequence and N-terminal of pcDNA3.1 site) and 5′-GGGGACCACTTTGTACAAAGAAAGCTGGGTCCTAGTCATCAGGCCTCTCTTCTGG-3′ (C-terminal reverse primer including complimentary attB sequence and complimentary C-terminal of A<sub>1</sub>AR). The 3xHA-A<sub>1</sub>AR coding sequence was subsequently cloned into the Gateway entry vector pDONR201 using the BP clonase enzyme mix (Invitrogen). Alanine amino acid substitutions were performed using oligonucleotides from GeneWorks (Hindmarsh, South Australia) (Supplemental Table 1) and QuickChange II site-directed mutagenesis kits (Stratagene, La Jolla, CA) according to the manufacturer’s instructions. Sequences of receptor clones were confirmed by automated sequencing as described previously (May et al., 2007). Receptor clones
were then sub-cloned into the Gateway destination vector pEF5/FRT/V5-DEST using the LR
clonase enzyme mix (Invitrogen).

Transfections and Cell Culture

DNA constructs in the pEF5/FRT/V5-DEST destination vector were stably transfected into Flp-
In™-CHO cell line as described previously (May et al., 2007). Briefly, Flp-In™-CHO cells were
transfected in serum and antibiotic-free DMEM with 0.7 μg of pEF5/FRT/V5-DEST vector
containing the wild-type (WT) or mutant 3xHA-A1AR constructs and 7 μg of pOG44 vector using
Lipofectamine 2000 (30 μL/25 cm² flask). Flp-In™-CHO cells stably expressing human A1AR
(untagged) were generated previously (Valant et al., 2014). Cells were selected and maintained in
DMEM containing 10% FBS and 600 μg/mL hygromycin B in a humidified environment at 37°C in
5% CO₂.

Radioligand binding assay

Cells were seeded at 40,000 cells/well into a transparent 96-well plate and incubated in a
humidified environment at 37°C in 5% CO₂. After 8 h, cells were washed with serum free DMEM
and maintained in serum free DMEM for approximately 18 h at 37°C in 5% CO₂ before assaying.
[^3H]DPCPX whole cell saturation binding assays on Flp-In™-CHO cells stably expressing A1AR
constructs were performed at 4°C for 3 h in a final volume of 100 μL 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 7.4) in the absence or presence of increasing concentrations of[^3H]DPCPX (0.1 nM-
10 nM).[^3H]DPCPX whole cell competition binding assays were performed under the same
conditions with ~1 nM[^3H]DPCPX and increasing concentrations of NECA (0.3 nM-30 μM) or
DPCPX (8-Cyclopentyl-1,3-dipropylxanthine; 0.1 nM-10 μM). Nonspecific binding was defined in
the presence of 1 μM SLV 320 (trans-4-[(2-Phenyl-7H-pyrrolo[2,3-d]pyrimidin-4-
yl)amino]cyclohexanol), a selective A₁AR antagonist (Kalk et al., 2007). Assays were terminated.
by washing twice with 100 μL cold phosphate buffered saline (PBS)/well, followed by the addition of 100 μL OptiPhase Supermix™ scintillation cocktail and bound radioactivity measured using a MicroBeta™ plate counter (PerkinElmer).

**cAMP accumulation assay**

Inhibition of forskolin-stimulated cAMP accumulation assays were performed as described previously (Baltos et al., 2016). Briefly, cells were seeded at 20,000 cells/well into a transparent 96-well plate and incubated in a humidified incubator at 37°C in 5% CO₂ overnight. Media was then replaced with 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, 5 mM HEPES, 0.1% bovine serum albumin, and 10 μM rolipram, pH 7.45) and incubated at 37°C for 30 min. Concentration response assays were performed by subsequent incubation of NECA (10 pM - 10 μM) and 3 μM forskolin for an additional 30 min at 37°C. Detection of cAMP was performed using AlphaScreen® cAMP Assay kits (PerkinElmer; Boston, MA) and fluorescence was measured with an EnVision® plate reader (PerkinElmer; Boston, MA) using standard AlphaScreen™ settings. Agonist concentration-response curves were normalized to the response mediated by 3 μM forskolin (0%) or buffer (100%) alone.

**Data Analysis**

All data were analysed using GraphPad Prism 6.03 (GraphPad Software, San Diego, CA). Relative receptor expression (Bₘₐₓ) and [³H]DPCPX equilibrium dissociation constants (Kₐ) were determined from saturation binding assays using the following equation:

\[
Y = \frac{B_{\text{max}}[A]}{[A] + K_D} + NS[A] 
\]

(Equation 1)
where Y is radioligand binding, B\textsubscript{max} is the total receptor density, [A] is the radioligand concentration, K\textsubscript{D} is the equilibrium dissociation constant of the radioligand, and NS is nonspecific radioligand binding.

[^3]H[DPCPX] competition binding curves were fitted to a one-site inhibitory mass action equation:

\[
Y = Bottom + \frac{Top - Bottom}{1 + 10^{(X - \text{LogIC}_{50})}}
\]

(Equation 2)

where Y is the specific binding and IC\textsubscript{50} is the concentration of ligand that displaces 50% of the radioligand. Top and bottom represent the maximal and minimal binding in the presence of the competitive ligand, respectively. Equilibrium dissociation constant, (K\textsubscript{i}) values were subsequently derived from inhibition binding experiments using the Cheng-Prusoff equation (Cheng and Prusoff, 1973):

\[
\frac{D}{K_{A}} = \frac{IC_{50}}{1 + \frac{[A]}{K_{D}}}
\]

(Equation 3)

NECA concentration-response curves were fitted to the following form of the operational model of agonism (Black and Leff, 1983):

\[
Y = \text{basal} + \frac{E_{m} - \text{basal}}{1 + \left(\frac{K_{A} + [A]}{\tau \times [A]}\right)^n}
\]

(Equation 4)

where E\textsubscript{m} is the maximal system response, A is the agonist concentration, \(\tau\) is an index of coupling efficiency (efficacy) of the agonist and is defined as RT/KE (where RT is the total concentration of receptors, i.e., B\textsubscript{max}, and KE is the concentration of agonist-receptor complex that yields half the E\textsubscript{m}), and n is the slope of the transducer function that links occupancy to response; the latter parameter was constrained to be shared across all datasets, as an extra-sum-of-squares (F test) determined that curve fitting was not improved by allowing different transducer slopes for different receptor mutations. K\textsubscript{A} values were constrained to their respective K\textsubscript{i} values estimated from[^3]H[DPCPX] competition binding studies. To account for effects of the expression level of each of the different mutant receptors on the observed efficacy of each agonist, the B\textsubscript{max} values determined
from saturation binding were used to normalize the $\tau$ values derived from the operational model analysis (Gregory et al., 2010); these values are reported as “corrected $\tau$ values”, $\tau_c$.

Statistical analysis was performed using a Student’s $t$-test or a one-way ANOVA analysis of variance with a Dunnett’s post-test as indicated was used to determine statistical differences ($P < 0.05$) where appropriate.

**Molecular Modeling**

*Homology Modeling of the A1AR*

The human A1AR and A2AAR share approximately 40% sequence identity and therefore A2AAR crystal structures are acceptable templates for A1AR homology models to gain a better understanding of ligand interactions at the A1AR. The sequence of the human A1AR was retrieved from the Swiss-Prot database. ClustalX software (Thompson et al., 1997) was used to align the A1AR sequence with the crystal structures of the antagonist and agonist-bound human A2AAR (PDB ID: 3EML (Jaakola et al., 2008) and 3QAK (Xu et al., 2011) for the inactive and partially active states, respectively). 3D structures of the A1AR were constructed with MODELLER v9.12 (Eswar et al., 2007). The conserved disulfide bond between residue C80$^{3.25}$ at the top of TM3 and C169$^{ECL2}$ and the ECL3 intra-loop disulfide bond between C260$^{ECL3}$ and C263$^{ECL3}$ present in the template structure were built and maintained as a constraint for geometric optimization. The best structure was selected based on the Modeller DOPE (Discrete Optimized Protein Energy) assessment score and visual inspection. Structures obtained were optimized using the Duan force field (Duan et al., 2003).

*Docking of adenosine receptor ligands*

Docking of DPCPX and NECA was performed using ICM version 3.8.0 (Molsoft L.L.C., La Jolla, CA). Potential binding sites were predicted using the ICM Pocket Finder algorithm (An et al., 2005; Abagyan and Kufareva, 2009). The docking search boundaries were defined by a box of 30 Å x 30
Å x 30 Å (the region in which 0.5 Å grid energy maps were generated), this size is large enough to encompass the potential orthosteric binding pocket and the extracellular region of the receptor. Docking thoroughness, representing the length of the docking simulation, used default settings. Ligand binding modes were ranked according to ICM score, which is inversely proportional to the number of favorable intermolecular interactions between the docked ligand and the receptor. The docking of each ligand was repeated five times and the conformation with the lowest ICM score selected for subsequent MD simulations.

Molecular Dynamics Simulations

MD simulations of the final complex were carried out with the NAMD2.10 (Phillips et al., 2005) package using the 3-site rigid water TIP3P model, CHARMM27 (MacKerell et al., 1998; Mackerell et al., 2004), and CGenFF (Vanommeslaeghe et al., 2010; Yu et al., 2012) v.3.0.1 force fields as described previously (Aksimentiev et al., 2012; Shonberg et al., 2013). The particle mesh Ewald (PME)(Essmann et al., 1995) method was used to evaluate electrostatic interactions. Each system contains an A1AR receptor, the ligand, a lipid bilayer containing ~220 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) molecules generated using the membrane plugin of the VMD software (v1.9.2) (Humphrey et al., 1996), and ~15800 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). Two energy minimization and equilibration periods were performed prior to the MD production run. The first involved energy minimization and a 15 ns equilibration of lipids tails. The second involved energy minimization and a 5 ns equilibration of the whole system, with 10 kcal mol\(^{-1}\)Å\(^{-2}\) harmonic-position restraints applied to all heavy atoms of the protein and ligands. Subsequently, the production run of the final unconstrained system was performed for 40 ns using a 2 fs integration time step in the NPT ensemble (310K, 1 bar). Coordinates were written to the output trajectory file every 10 ps. VMD v1.9.2 was used for the visualization and analysis of the residue-ligand contacts through the course of each simulation using in-house scripts. The percentage total MD time that residues were involved in hydrogen bonding...
and/or found within 3.5 Å of DPCPX or NECA heavy atoms was quantified for each MD simulation. All figures were generated using PYMOL.

Results

The 3xHA-A1AR has comparable pharmacology to the human A1AR

In this study, the role of ECL2 residues on agonist binding and function was investigated using human A1AR constructs with an N-terminal triple HA epitope tag (3xHA-A1AR). Previous studies on other GPCRs have shown that the addition of an N-terminal HA-tag generally does not interfere with receptor pharmacology (Sromek and Harden, 1998; Leach et al., 2011; Schiedel et al., 2011). To ensure the pharmacology of the 3xHA-A1AR was equivalent to the untagged human A1AR, we compared agonist and antagonist affinity estimated between the two constructs. In homologous competition binding studies, the N-terminal 3xHA tag significantly reduced receptor expression (B_max) (A1AR B_max = 5343 ± 234.6, 3xHA-A1AR B_max = 2022 ± 155; n = 3 – 6; P < 0.05, Student's t-test), however, DPCPX affinity was unaffected (A1AR pKi = 8.84 ± 0.08, 3xHA-A1AR pKi = 8.96 ± 0.11; n = 3 – 6; P > 0.05, Student's t-test). In heterologous competition binding studies, NECA affinity was also unaffected (A1AR pKi = 6.48 ± 0.01, 3xHA-A1AR pKi = 6.59 ± 0.04; n = 3 – 44; P > 0.05, Student's t-test). Therefore despite having decreased cell surface expression, the N-terminal 3xHA epitope tagged human A1AR has equivalent affinity with the orthosteric ligands to the untagged human A1AR.

Cell surface expression of human 3xHA-A1AR ECL2 alanine mutants

The generalized numbering scheme proposed by Ballesteros and Weinstein was used for transmembrane residues (Ballesteros and Weinstein, 1995). Alanine scanning mutations were performed within ECL2 of the A1AR, with the exception of C169_ECL2. Residues C80_ECL2 and C169_ECL2 are predicted to form a conserved disulphide bond, and alanine substitution of C169_ECL2 has previously been shown to abolish [3H]DPCPX specific binding (Scholl and Wells, 2000;
Single alanine mutations were performed, with the exception of S150ECL2A + V151ECL2A (SV150ECL2AA) and M162ECL2A + G163ECL2A + E164ECL2A (MGE162ECL2AAA), for which the double and triple mutations were similarly expressed (Fig. 1A). Cell surface expression of each mutant 3xHA-A1AR stably expressed in Flp-In™-CHO cells was determined from whole cell [³H]DPCPX saturation binding (Fig. 1B, Fig. 2A). No [³H]DPCPX specific binding could be detected at the F171ECL2A mutation, however subsequent detection of the 3xHA tag with an ELISA found that the cell surface expression of the F171ECL2A receptor was not significantly different to that of the WT A1AR (P > 0.05, unpaired t test; Supplemental Fig. 1). Orthosteric ligand affinity is predicted to be significantly influenced by the F171ECL2A mutation as the corresponding residue at the A2AAR, F168ECL2, has been previously shown to form a π-stacking interaction with the antagonist ZM241385 (4-(2-[7-amino-2-(2-furyl)-[1,2,4]triazolo-[2,3-a][1,3,5]triazin-5-ylamino]ethyl)-phenol) and agonist NECA in A2AAR crystal structures (Jaakola et al., 2008; Liu et al., 2012). As such, the lack of [³H]DPCPX specific binding observed for F171ECL2A is likely due to a significant decrease in [³H]DPCPX affinity. Of the remaining 25 mutant 3xHA-A1ARs investigated, 17 mutant 3xHA-A1AR Flp-In™-CHO cell lines had significantly reduced cell surface receptor expression compared to the WT 3xHA-A1AR Flp-In™-CHO cell line (Fig. 1B, Supplemental Table 2).

**ECL2 alanine substitutions influence A1AR orthosteric ligand affinity**

The affinity of the orthosteric antagonist [³H]DPCPX and orthosteric agonist NECA was determined from whole cell saturation and competition binding assays, respectively (Fig. 2A, Fig. 2B, Table 1). When compared to the WT 3xHA-A1AR, alanine substitution of multiple residues (N159ECL2, S161ECL2, I167ECL2, E172ECL2, I175ECL2 and M177ECL2) located in the middle to end of ECL2, significantly decreased [³H]DPCPX affinity (pKᵩ) (Fig. 3A). The same residues had a similar or greater effect on NECA affinity (pKᵦ) (Fig. 3B), suggesting that these ECL2 residues facilitate, through direct or indirect interactions, the binding of [³H]DPCPX and NECA at the
A1AR. Three additional ECL2 mutations, W146\textsuperscript{ECL2}A, L149\textsuperscript{ECL2}A and E170\textsuperscript{ECL2}A, had a modest effect on NECA affinity (Fig. 3B, Table1).

**ECL2 alanine substitutions influence A1AR orthosteric agonist efficacy**

To investigate the influence of ECL2 on A1AR agonist efficacy, NECA-mediated inhibition of forskolin-stimulated cAMP accumulation was quantified in WT and mutant 3xHA-A1AR Flp-In\textsuperscript{TM}-CHO cell lines (Fig. 2C). The NECA-mediated inhibition of cAMP accumulation was comparable in the absence and presence of adenosine deaminase, suggesting minimal influence of endogenous adenosine (unpublished results). Furthermore, we have recently demonstrated that the Flp-In\textsuperscript{TM}-CHO cell line stably expressing the wild-type A1AR was not associated with measureable constitutive activity (Vecchio et al., 2016). Similarly, comparison of the cAMP accumulation stimulated by forskolin (3 \(\mu\)M) found no evidence for A1AR constitutive activity in Flp-In\textsuperscript{TM}-CHO cells stably expressing mutant A1ARs (unpublished results). An operational measure of efficacy (\(\log\tau\)), corrected for relative receptor expression levels, was used to quantify efficacy changes at mutant 3xHA-A1ARs compared to the WT 3xHA-A1AR. Negligible NECA-mediated inhibition of cAMP accumulation was observed in the Flp-In\textsuperscript{TM}-CHO cell line expressing the 3xHA-A1AR(F171\textsuperscript{ECL2}A) (data not shown). Interestingly, the cluster of 7 residues at the C-terminal end of the ECL2 that significantly reduced NECA affinity had no impact on NECA efficacy (\(\log\tau\)). NECA efficacy was significantly reduced, however, at 5 of the mutant 3xHA-A1ARs (Fig. 4, Table 1). Of note, NECA efficacy was significantly reduced upon alanine substitution of N148\textsuperscript{ECL2}, E153\textsuperscript{ECL2}, R154\textsuperscript{ECL2} and W156\textsuperscript{ECL2}; mutations that had no significant effect on NECA affinity. In contrast to the effect of L149\textsuperscript{ECL2}A in improving NECA affinity, the same mutation decreased NECA efficacy.

**Ligand Docking and Molecular Dynamics Simulations**
Inactive and active-like homology models of the human A_1AR were generated based on the antagonist ZM241385 and agonist UK432097 (6-[2,2-di(phenyl)ethylamino]-9-[(2R,3R,4S,5S)-5-(ethylcarbamoyl)-3,4-dihydroxyoxolan-2-yl]-N-[2-[(1-pyridin-2-yl)piperidin-4-yl]carbamoylamino]ethyl]purine-2-carboxamide) occupied crystal structures of the human A_2AAR (PDB ID: 3EML and 3QAK), respectively (Jaakola et al., 2008; Xu et al., 2011). Ligand docking and MD simulations were performed using our inactive and active-like A_1AR homology models to assist with the interpretation of mutagenesis findings. The ICM PocketFinder algorithm identified three potential binding pockets within our A_1AR homology models. DPCPX and NECA were docked into the pocket located within the TM bundle, however the size of docking box was adjusted to be large enough to encompass the extracellular region of the receptor. Docking simulations of DPCPX resulted in a single cluster within the TM bundle, where DPCPX formed a \( \pi \)-stacking interaction with F171\textsuperscript{ECL2} (Fig. 5A); this key interaction was maintained during a 40 ns MD simulation (Table 2). Furthermore, the key residue, N254\textsuperscript{6.55}, involved in hydrogen bonding interactions in both antagonist and agonist bound A_2AAR crystal structures, formed a hydrogen bond with DPCPX during the MD simulation (Table 2). E172\textsuperscript{ECL2}, another key residue involved in the formation of the DPCPX binding pocket, that is, found within 3.5 Å of DPCPX during the MD simulation, also decreased \([^3]H\)DPCPX affinity when mutated to alanine (Table 2). The root-mean-square-deviation of DPCPX compared to its final pose was calculated. Over the course of the simulation, DPCPX remained relatively stable after 12 ns (Supplemental Fig. 2).

Interestingly, molecular docking of NECA revealed two main clusters amongst the top 40 docking poses, one within the TM bundle (‘Site 1’) and the other within the extracellular region (‘Site 2’) (Fig. 5B). Similar findings were also observed when we repeated the molecular docking using the endogenous agonist adenosine instead of NECA (Supplemental Fig 3). The NECA poses found within the TM bundle (Site 1) were located within the predicted A_1AR orthosteric agonist site. Within Site 1, poses could be further divided into two sub-clusters. One sub-cluster had a similar
orientation of the adenine ring to that found within the NECA bound A_{2A}AR crystal structure and was relatively stable during our 40 ns MD simulation (Supplemental Fig. 2). During this simulation, NECA predominantly formed hydrogen bond interactions with the residues T91^{3.36}, N254^{6.55} and T277^{7.42}. The only ECL2 residue located within 3.5 Å of NECA during this MD simulation was F171^{ECL2} (Table 2). The alternative cluster of NECA Site 1 poses was not considered for MD simulations, as the adenine ring was inverted relative to the position of NECA bound within the A_{2A}AR crystal structure.

NECA poses that docked within the A_{1}AR extracellular region (Site 2) formed hydrogen bonds with the three extracellular loops and residues at the top of TM6 and TM7. A 40 ns MD simulation was performed on the highest ranked (lowest ICM score) pose within the extracellular vestibule. Within this MD simulation, NECA was relatively stable and formed hydrogen bonds with a number of residues including E170^{ECL2}, E172^{ECL2}, N254^{6.55}, K265^{ECL3}, P266^{ECL3} and T270^{7.35} (Table 2; Supplemental Fig. 2). ECL2 residues located within 3.5 Å of NECA during this MD simulation were E170^{ECL2}, F171^{ECL2}, E172^{ECL2} and M177^{ECL2} (Table 2). These findings are consistent with mutagenesis data that suggest each of these residues are involved in the binding of NECA to the A_{1}AR (Table 1).

A main pose of each ligand, which formed key interactions as determined by the interaction frequency analysis of the MD trajectory, was obtained from each 40 ns MD simulation (Fig. 6, Fig. 7, Data Supplement 1-3). The purine rings for the DPCPX and NECA (Site 1) poses overlap, stabilized by a H-bond with N254^{6.55} and π-stacking interactions with F171^{ECL2} (Supplemental Fig. 4). Residues within the extracellular vestibule, top of TM6 (L250^{6.51}, L253^{6.54} and T257^{6.58}) and E172^{ECL2} surrounded the DPCPX cyclopentyl ring, whereas the NECA ribose moiety made contacts with residues deeper within the TM bundle (V87^{3.32}, L88^{3.33}, Q92^{3.37}, M180^{5.40}, and W247^{6.48}). NECA was further anchored by a predicted hydrogen-bond with T91^{3.36} (Fig. 7A, Fig. 7B).
Therefore, the residues predicted to form the NECA Site 1 binding pocket involve conserved residues between the A₁AR and A₂AAR, V87<sup>3.32</sup>, L88<sup>3.33</sup>, T91<sup>3.36</sup>, Q92<sup>3.37</sup>, M180<sup>5.40</sup>, W247<sup>6.48</sup>, L250<sup>6.51</sup> and N254<sup>6.55</sup>.0.08em These residues are predicted to be involved in conformational rearrangements of TM3, 5, 6 and 7 upon receptor activation and facilitate the binding of orthosteric agonists (Supplemental Fig. 4)(Xu et al., 2011; Lebon et al., 2011). The main NECA binding pose within Site 2 was predicted to be stabilized by H-bond interactions between the adenine ring and E170<sup>ECL2</sup>, E172<sup>ECL2</sup>, S267<sup>ECL3</sup>, and T270<sup>7.35</sup> as well as a H-bond between the 2′-OH group of ribose ring and K265<sup>ECL3</sup> (Fig. 7C, Fig. 7D). The NECA Site 2 binding pocket was also closely packed by F171<sup>ECL2</sup>, M177<sup>ECL2</sup> and T257<sup>6.58</sup> side chains. Interestingly, the NECA (Site 2) and DPCPX poses were not topographically distinct and instead predicted to partially overlap.

*Alanine substitution of predicted binding pocket residues from molecular modeling impact*

**NECA affinity and/or efficacy**

Results from the NECA Site 1 MD simulations found T91<sup>3.36</sup>, F171<sup>ECL2</sup> and W247<sup>6.48</sup> located within 3.5 Å of NECA heavy atoms for at least 60% of the total MD simulation time. Whereas the NECA Site 2 MD simulation found E172<sup>ECL2</sup>, K265<sup>ECL3</sup> and T270<sup>7.35</sup> located within 3.5 Å of NECA heavy atoms for at least 60% of the total MD simulation time. The corresponding conserved residues T91<sup>3.36</sup>, F171<sup>ECL2</sup> and E172<sup>ECL2</sup> directly interact with NECA within the A₂AAR crystal structure. Furthermore, the involvement of the TM residue T91<sup>3.36</sup> on A₁AR agonist binding has been confirmed previously through mutagenesis studies (Rivkees et al., 1999). The conserved tryptophan at position 6.48 has been suggested to be involved in the activation process of GPCRs (Shi et al., 2002; Kobilka et al., 2007; Stoddart et al., 2014). Given the known role of T91<sup>3.36</sup> and W247<sup>6.48</sup> in A₁AR orthosteric binding, these residues were not investigated further in the current study. However, the influence of the predicted NECA Site 2 residues, K265<sup>ECL3</sup> and T270<sup>7.35</sup>, on agonist binding or function has not been investigated at the human A₁AR. Therefore, we investigated the influence of single point alanine substitutions of K265<sup>ECL3</sup>A and T270<sup>7.35</sup>A, on A₁AR
pharmacology. Consistent with DPCPX binding within the TM bundle, neither mutation significantly influenced receptor expression (Fig. 8A) or DPCPX affinity (Fig. 8B). In contrast, alanine substitution of T270<sup>7,35</sup> caused a significant decrease in NECA affinity (Fig. 8C). Furthermore, both mutations significantly decreased NECA efficacy (Fig. 8D). These data suggest that residues in the extracellular region of the A<sub>1</sub>AR play an important role in both agonist binding and function.
Discussion

The A₁AR represents a potential therapeutic target for a number of conditions. A detailed understanding of the structure-function relationships at the A₁AR can facilitate the rational design of more potent and selective A₁AR ligands. Since a high resolution structure of the A₁AR bound to different classes of ligand is not yet available, less direct methods such as site-directed mutagenesis and molecular modeling that can probe the structure-function properties underlying A₁AR orthosteric ligands remain extremely valuable. Given that ECL2 has been suggested to have a critical role in the binding and function of orthosteric ligands at a range of GPCRs (Wheatley et al., 2012), we performed alanine scanning mutagenesis of this region combined with molecular modeling to identify the role of this domain on orthosteric ligand pharmacology. Moreover, key residues predicted from MD to be involved orthosteric agonist function were mutated, and the effects of the mutations validated the importance of these residues. Our A₁AR-ECL2 mutagenesis data are in good agreement with the binding modes of DPCPX and NECA from molecular modeling, identifying a key role for ECL2 in A₁AR orthosteric ligand binding and function. However, our data also suggest that the orthosteric agonist, NECA, recognizes a binding site in an extracellular vestibule that may represent a transition pose prior to entry into the canonical orthosteric site. This vestibular region may represent part of an allosteric binding site for A₁AR modulators, and this is explored in greater detail in our accompanying article.

Previous A₁AR site-directed mutagenesis studies have predominantly focused on TM domains, with residues in TM 1, 2, 3, 4, 5, and 7 implicated in the binding of orthosteric agonists and antagonists (Olah et al., 1994; Townsend-Nicholson and Schofield, 1994; Barbhaiya et al., 1996; Rivkees et al., 1999; Fredholm et al., 2001). Until now, the involvement of ECL2 on orthosteric ligand binding and function has not been systematically investigated at the A₁AR. However, a critical role for this domain on orthosteric pharmacology has been identified from mutagenesis studies on other GPCRs, such as the A₃AR, dopamine D₂ receptor, M₂ and M₃ muscarinic receptors, V₁a vasopressin receptor...
and glucagon-like peptide-1 receptor (Howl and Wheatley, 1996; Gao et al., 2002; Shi and Javitch, 2004; Duong et al., 2005; Avlani et al., 2007; Scarselli et al., 2007; Koole et al., 2012). It is suggested that orthosteric ligand binding to an unoccupied GPCR, which has a more exposed ECL2, can induce a conformational change, causing ECL2 to close over the entrance to the binding crevice and thereby stabilize the ligand within the orthosteric pocket (Banères et al., 2005; Unal et al., 2010). Supporting this notion, ECL2 contributes to the orthosteric pocket in A2AR crystal structures. The short helical domain within the A2AR-ECL2 is oriented towards the TM core region and interacts directly with agonists and antagonists in the orthosteric site (Jaakola et al., 2008; Lebon et al., 2011; Xu et al., 2011; Doré et al., 2011; Congreve et al., 2012; Hino et al., 2012; Liu et al., 2012; Lebon et al., 2015). Molecular modeling performed within the current study, also suggests the short helical domain within the A1AR-ECL2 forms the upper region of the principal orthosteric pocket. The main pose from MD simulations performed on DPCPX and NECA docked within the TM bundle found two ECL2 residues, F171 and E172, located within 3.5 Å of the orthosteric ligand (Fig. 6, Fig. 7). Supporting a role of these residues in DPCPX binding at the A1AR, a decrease in [3H]DPCPX and NECA affinity was observed for the E172ECL2A and no [3H]DPCPX binding was observed at the F171ECL2A mutation.

Docking of DPCPX and NECA into homology models based on inactive and active-like A2AR crystal structures, respectively, revealed overlapping binding poses, with NECA (bound to Site 1) making additional contacts with residues deeper within the TM bundle than DPCPX. Previous studies found that TM3 residues differentially impacted A1AR orthosteric antagonist versus agonist binding. Mutation of L883.33A caused a substantial reduction of both agonist and antagonist affinity at the A1AR, whereas and V873.32A has been shown to decrease antagonist affinity alone and Q923.37A has been shown to perturb agonist affinity only (Rivkees et al., 1999). In the main poses after MD simulation, V873.32 and L883.33A were predicted to have hydrophobic interactions with both DPCPX and NECA, while Q923.37 only interacted with NECA (Site 1) (Supplemental Fig. 4).
Therefore, the key interactions predicted from our molecular modelling are in agreement with previous mutagenesis studies and further support a deeper orthosteric-binding pose for NECA (Site 1) relative to the antagonist, DPCPX. It should be noted, however, that the single identified stable pose of the antagonist, DPCPX, is higher-placed such that it overlaps with both NECA-binding clusters i.e., within the orthosteric agonist pocket (Site 1) and the extracellular vestibule (Site 2) to antagonize agonist access.

ECL2 has also been suggested to facilitate ligand access from the extracellular space into the orthosteric TM binding cavity. For instance, in unbiased long time scale MD simulations, β-adrenoceptor orthosteric ligands recognized an intermediate binding site within the extracellular vestibule during the transition from the extracellular space into the TM bundle (Dror et al., 2011). Furthermore, the importance of ECL2 flexibility and conformational integrity was highlighted in a study on the M2 muscarinic acetylcholine receptor, where a reduction in ECL2 flexibility due to the introduction of a disulfide bond between ECL2 and TM7, caused a significant decrease in orthosteric and allosteric ligand affinity (Avlani et al., 2007). Supporting a role of ECL2 in facilitating ligand entry into the orthosteric site, our docking of NECA in the active-like A1AR homology model found 31 of the top 40 poses recognized a common site within the extracellular vestibule. The main pose from the MD simulation performed on NECA docked within this site had hydrogen-bonding interactions between the ligand and E170$^{\text{ECL2}}$, E172$^{\text{ECL2}}$, K265$^{\text{ECL3}}$, S267$^{\text{ECL3}}$ and T270$^{7,35}$. ECL2 residues located within 3.5 Å of this NECA pose were E170$^{\text{ECL2}}$, F171$^{\text{ECL2}}$, E172$^{\text{ECL2}}$ and M177$^{\text{ECL2}}$. Two of these residues, F171$^{\text{ECL2}}$ and E172$^{\text{ECL2}}$ are also involved in the formation of the orthosteric pocket within the TM bundle. Nonetheless, the existence of the Site 2 binding pocket is supported by ECL2 alanine scanning mutagenesis, which found a significant decrease in NECA affinity at the A1AR mutations E170$^{\text{ECL2}}$A, E172$^{\text{ECL2}}$A and M177$^{\text{ECL2}}$A. Furthermore, alanine mutation of residues predicted from our MD simulations to interact with NECA at this extracellular site, K265$^{\text{ECL3}}$ and T270$^{7,35}$, caused a significant decrease in NECA
affinity and/or efficacy. Of note, $\beta_2$-adrenoceptor residues that form key interactions with orthosteric ligands within the extracellular vestibule, $Y_{308}^{7.35}$ and $F_{193}^{ECL2}$ (Dror et al., 2011), correspond to $T_{270}^{7.35}$ and $F_{171}^{ECL2}$ identified within our study. Initial recognition within the extracellular vestibule (Site 2) may act as a ‘selectivity filter’ for GPCRs. The relatively open nature of the extracellular vestibule may lead to Site 2 being more populated by a range of ligands, however only those that form the appropriate metastable poses will eventually transition into Site 1. In contrast, ligands that do not form these metastable interactions within Site 2 will leave this site and dissociate from the receptor. Collectively, these data add weight to the notion that the ECL2 of numerous class A GPCRs contributes to an extracellular vestibule that can bind small molecules, including orthosteric agonists as well as allosteric modulators.

Within this study, alanine mutation of a number of residues in the middle of ECL2, which are predicted from molecular modeling to be distinct from the orthosteric site, caused a significant decrease in orthosteric ligand affinity and/or efficacy. This is perhaps unsurprising given the flexible nature of ECL2 and therefore the spectrum of conformations it likely assumes. Furthermore, ECL2 has not been well defined within $A_{2A}$AR crystal structures, with many residues not resolved within this region. However, ECL2 residues suggested from alanine scanning mutagenesis to be involved in orthosteric ligand binding may influence the energetics of the unbound receptor conformations and/or facilitate the transition of ligands from the extracellular space into the orthosteric site. Indeed, we identified a cluster of four residues at the TM4 end of ECL2 that perturbed NECA efficacy in the absence of an effect on affinity, indicating that ECL2 is involved in the stabilization of $A_1$AR active states. These findings are in line with previous studies of other family A GPCRs where mutations within ECL2 perturbed orthosteric agonist efficacy (May et al., 2007; Scarselli et al., 2007; Gregory et al., 2010; 2012; Wifling et al., 2015). At a number of GPCRs, including the $M_2$ muscarinic acetylcholine and glucagon-like peptide-1 receptor, ECL2 differentially influences orthosteric agonist efficacy depending upon the signalling pathway.
assessed (Gregory et al., 2010; 2012; Koole et al., 2012), and as such is thought to play a key role in the stabilization of different active states that contribute to the phenomenon of biased agonism. In the future, it will be pertinent to explore the influence of these ECL2 residues on the bias profile of A1AR agonists.

Informed by our iterative approach that combined site-directed mutagenesis and molecular modeling with rigorous analysis delineating effects of select A1AR residues on orthosteric affinity and efficacy, we have demonstrated a key role for ECL2 in A1AR agonist and antagonist binding and function. Our analysis indicated that agonist ligand affinity and efficacy determinants could be clearly delineated into two major clusters. In addition to making direct contact with ligands bound to the transmembrane bundle, we propose that ECL2 forms an extracellular vestibule that is recognized by, and facilitates the binding of, orthosteric agonists. It would be of interest to determine the extent to which this vestibule also contributes to the actions of A1AR allosteric ligands.
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Authorship contributions

Participated in research design: ATNN, LTM, AC

Conducted experiments: ATNN, JB, LLM, KJG

Performed data analysis: ATNN, TDN, TT

Wrote or contributed to the writing of the manuscript: ATNN, PMS, KJG, PJW, LTM, AC
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Footnotes

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FIGURE LEGENDS

**Fig. 1.** A) Diagram highlighting alanine mutations within the human A1AR. Single alanine substitutions are highlighted in red whereas double and triple mutations are blue. B) Receptor expression (B_max) of WT and mutant 3xHA-A1ARs stably expressed in Flp-In™-CHO cells. B_max values are expressed as a percentage of WT and represent the mean ± S.E.M of at least 3 experiments performed in duplicate. *P < 0.05 compared to WT (one-way analysis of variance, Dunnett’s post hoc test). ND, not determined as no specific radioligand binding detected.

**Fig. 2.** Radioligand binding and cAMP accumulation data for Flp-In™-CHO cells stably expressing WT or selected mutant 3xHA-A1ARs. A) [3H]DPCPX whole cell saturation binding, B) [3H]DPCPX whole cell competition binding and C) cAMP accumulation. Data are mean ± S.E.M from at least 3 experiments conducted in duplicate. Where error bars are not shown they lie within the dimensions of the symbol.

**Fig. 3.** The change in affinity of A) DPCPX (ΔpKD) and B) NECA (ΔpKi) at mutant 3xHA-A1ARs relative to WT. Data represents the mean ± S.E.M of at least 3 experiments performed in duplicate. *P < 0.05 compared to WT (one-way analysis of variance, Dunnett’s post hoc test).

**Fig. 4.** The change in signaling efficacy of NECA (Δlogτc) at mutant 3xHA-A1ARs relative to WT. Data represent the mean ± S.E.M of at least 3 experiments performed in duplicate. *P < 0.05 compared to WT (one-way analysis of variance, Dunnett’s post hoc test).

**Fig. 5.** Docking of DPCPX and NECA into A1AR homology models. A) The top 20 DPCPX (sticks; carbon, nitrogen, oxygen and polar hydrogen atoms are colored in cyan, blue, red and white, respectively) poses docked into the inactive A1AR homology model (grey ribbons). F171ECL2,
predicted to form a key $\pi$-stacking interaction with DPCPX, is shown as blue sticks. **B**) The top 40 NECA (sticks; carbon, nitrogen, oxygen and polar hydrogen atoms are colored in green, blue, red and white, respectively) poses docked into the active-like A$_1$AR homology model (light blue ribbons). Residues predicted to be involved in hydrogen bond and $\pi$-stacking interactions are shown as blue sticks.

**Fig. 6.** Predicted binding mode of DPCPX (cyan sticks) at the inactive A$_1$AR homology model (grey ribbons). Residues that significantly influenced DPCPX affinity are shown as yellow (<5 fold change) and orange (between 5-10 fold change) sticks. Key residues predicted to form hydrogen bond (N254$^{6.55}$) or $\pi$-stacking (F171$_{\text{ECL2}}$) interactions with DPCPX are shown as blue sticks.

**Fig. 7.** Predicted binding mode of NECA (green sticks) at the partially active A$_1$AR homology model (light blue ribbons) located in the orthosteric site (Site 1; **A** and **B**) and extracellular vestibule (Site 2; **C** and **D**). Residues that significantly influenced NECA affinity (**A**, **C**) or efficacy (**B**, **D**) are shown as yellow (<5 fold change), orange (between 5-10 fold change) and red (>10 fold change) sticks. Key residues predicted to form hydrogen bond interactions with NECA are shown as blue sticks.

**Fig. 8.** The influence of alanine substitution on residues predicted from MD simulations to be involved Site 2 NECA binding. **A**) Receptor expression ($B_{\text{max}}$) of WT and mutant 3xHA-A$_1$ARs stably expressed in Flp-In$^{\text{TM}}$-CHO cells. $B_{\text{max}}$ values are expressed as a percentage of WT. The change in [$^3$H]DPCPX affinity (**B**), NECA affinity (**C**) and NECA efficacy (**D**) for mutant 3xHA-A$_1$ARs relative to WT. Data represents the mean ± S.E.M of 3 experiments performed in duplicate. *P < 0.05 compared to WT (one-way analysis of variance, Dunnett’s post hoc test).
Table 1. Orthosteric whole cell equilibrium binding and agonist operational efficacy parameters at WT and mutant A<sub>1</sub>ARs mutant 3xHA-A<sub>1</sub>ARs stably expressed in Flp-In<sup>TM</sup>-CHO cells. pK<sub>D</sub> and pK<sub>I</sub> are the negative logarithm of the radioligand [³H]DPCPX and NECA equilibrium dissociation constants, respectively. Log<sub>c</sub> is logarithm of operational efficacy parameter for NECA determined using operational model of agonism, where NECA pK<sub>I</sub> values were constrained to those determined from the radioligand binding assays. Values represent the mean ± S.E.M. from the indicated number of individual experiments (n) performed in in duplicate.

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<td>pK&lt;sub&gt;I&lt;/sub&gt; (n)</td>
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*Significantly different from WT value (p < 0.05, one-way ANOVA with Dunnet’s post-hoc test)

NA, not applicable (no competition with the radioligand or no inhibition of cAMP)
Table 2. A1AR residues that interact with DPCPX or NECA through the course of each MD simulations. Values represent the percentage of the MD simulation time (40 ns) that the residue and ligand heavy atoms are within 3.5 Å of one another. Residues were considered to be interacting with the ligand if they were in contact with the ligand for >5% of simulation time. Hydrogen-bond interactions (in bracket) were defined with the donor-acceptor distance < 3.5 Å, and an angle cutoff of 20° and displayed as percentage of MD simulation time. All values were calculated for 1 frame per 10 ps using VMD v1.9.2.

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<td>Q92</td>
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L-RS: hydrogen-bond interaction between ligand (donor) and residue side chain (acceptor)
RS-L: hydrogen-bond interaction between residue side chain (donor) and ligand (acceptor)
L-RM: hydrogen-bond interaction between ligand (donor) and residue main chain (acceptor)
RM-L: hydrogen-bond interaction between residue main chain (donor) and ligand (acceptor)
Fig. 3

A

$\Delta pK_D$ DPCPX

B

$\Delta pK_I$ NECA
Fig. 4

Δlogτ_c NECA
