THE EFFECT OF ALLOSTERIC MODULATORS ON THE KINETICS OF AGONIST-G PROTEIN-COUPL ED RECEPTOR INTERACTIONS IN SINGLE LIVING CELLS.

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ALLOSTERIC MODULATION OF AGONIST KINETICS IN SINGLE CELLS

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Abbreviations: GPCR, G protein-coupled receptors; A_1AR, adenosine A_1 receptor; A_3AR, adenosine A_3 receptor; CHO-K1, Chinese hamster ovary; HBSS, HEPES-buffered saline; DMEM, Dulbecco’s modified Eagle's medium; FCS, fetal calf serum; ERK1/2, extracellular signal-regulated kinase 1 and 2; NECA, 5’-(N-ethyl carboxamido)adenosine; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; FCS, fluorescence correlation spectroscopy.
ABSTRACT

Allosteric binding sites on adenosine -A₁ and -A₃ receptors represent attractive therapeutic targets for amplifying, in a spatially and temporally selective manner, the tissue protective actions of endogenous adenosine. This study has directly quantified the kinetics of agonist/GPCR interactions at the single cell level, reflecting the physiological situation where intracellular signalling proteins can exert major allosteric effects on agonist-receptor interactions. The association and dissociation rate constants at both A₁ and A₃ receptors and therefore the affinity of the fluorescent adenosine derivative, ABA-X-BY630, were concentration-independent. The equilibrium dissociation constant of ABA-X-BY630 at A₁ and A₃ receptors was approximately 50 nM and 10 nM respectively; suggesting that, even in live cells, low agonist concentrations predominantly detect high affinity receptor states. At A₁ receptors, the dissociation of ABA-X-BY630 (30 nM) was significantly faster in the absence (kₐₒₙ=1.95±0.09 min⁻¹) as compared to the presence of the allosteric enhancer PD81,723 (10 μM; kₐₒₙ=0.80±0.03 min⁻¹) and allosteric inhibitor VUF5455 (1 μM; kₐₒₙ=1.48±0.16 min⁻¹). In contrast, ABA-X-BY630 dissociation from A₃ receptors was significantly slower in the absence (kₐₒₙ=0.78±0.18 min⁻¹) as compared to the presence of the allosteric inhibitors, VUF 5455 (1 μM; kₐₒₙ=3.15±0.12 min⁻¹) and PD81,723 (10 μM; kₐₒₙ=2.46±0.18 min⁻¹). An allosteric mechanism of action has previously not been identified for PD81,723 at the A₃ receptor or VUF5455 at the A₁ receptor. Furthermore, the marked enhancement in fluorescent agonist dissociation by VUF5455 in living cells contrasts previous observations from broken cell preparations and emphasizes the need to study the allosteric regulation of agonist binding in living cells.
INTRODUCTION

G protein-coupled receptors (GPCRs) represent the largest superfamily of cell surface receptors. Heterotrimeric ligand/GPCR/G protein complexes engender enormous diversity within cell signaling and as such, a large proportion of current pharmaceuticals target GPCRs (Overington et al., 2006). The majority of these drugs mediate their action through the primary, endogenous ligand binding site, which for GPCRs is known as the orthosteric binding site. However, GPCRs also contain allosteric binding sites; that is, any receptor binding site that is topographically distinct from the orthosteric site (May et al., 2007b). GPCR signaling, even at the simplest level, is governed by allosteric interactions with intracellular signaling proteins. Agonist binding within the extracellular domain promotes stabilization of GPCR conformations that preferentially interact with intracellular signaling proteins (Kenakin, 2002). GPCRs can also interact with transmembrane proteins and allosteric small molecules. These interactions stabilize an alternative subset of receptor conformations which in turn can cause a change in the rate of orthosteric ligand association and/or dissociation (May et al., 2007b). As a result, a wealth of information with respect to the conformational changes occurring within the receptor binding site can be obtained through assessing ligand binding kinetics under different conditions. However, the rapid nature of agonist kinetics at non-peptide GPCRs in the presence of the active G protein cycle has largely prevented any direct investigation of the binding kinetics of these agonists in living cells.

Over recent years, drug design targeted towards allosteric binding sites on GPCRs has gained momentum within drug discovery. This approach has a number of potential advantages, including the selective manipulation of GPCRs that contain high sequence homology within the orthosteric domain across receptor subtypes and the ability to maintain
the spatial and temporal signaling profile of the endogenous ligand; only enhancing or inhibiting receptor activity where and when the endogenous agonist is present (Birdsall et al., 1996). A fundamental method to detect GPCR allosterism has been to observe a change in the dissociation kinetics of an orthosteric ligand in the presence of a putative allosteric modulator (Christopoulos and Kenakin, 2002). The mechanism of action for the first identified GPCR allosteric enhancers, a series of adenosine A1 receptor (A1AR) selective 2-amino-3-benzoylthiophene derivatives including PD81,723, was confirmed through detecting a decrease in the rate of agonist dissociation (Bruns and Fergus, 1990). This method was used more recently to identify adenosine A3 receptor (A3AR) allosteric modulators, including VUF5455 (Gao et al., 2001).

To date, the majority of dissociation kinetic assays involve membrane-based radioligand binding, which has a number of limitations. For example, radioligand dissociation is generally measured in the presence of a saturating concentration of competitive orthosteric ligand (Avlani et al., 2004; Bruns and Fergus, 1990; Christopoulos et al., 1997; Dowling and Charlton, 2006; Ellis et al., 1991; Gao et al., 2001; Lazareno and Birdsall, 1995; Lee and el-Fakahany, 1991; May et al., 2007a; Springael et al., 2006). This requires the assumption that the second ligand does not alter radioligand dissociation. While consistent within a theoretical framework describing competitive interactions between compounds at a monomeric receptor, more complex interactions resulting from multistep ligand binding (Ilien et al., 2009; Swaminath et al., 2004) or receptor dimerization (Christopoulos and Kenakin, 2002; Han et al., 2009; Springael et al., 2006) could lead to a change in the radioligand dissociation rate. In addition, the absence of the G protein cycle in membrane-based assays generates a large bias for an agonist radioligand to detect the active, G protein-coupled, receptor conformation. Finally, membrane-based radioligand binding
assays lack the temporal resolution required to detect rapid changes in ligand binding. Taken together, these methodological limitations have restricted both qualitative and quantitative investigations of agonist kinetics either alone or in the presence of allosteric modulators at non-peptide GPCRs in living cells.

This study focused on visualizing and quantifying, at the single cell level, the binding kinetics of an adenosine receptor agonist in the absence and presence of allosteric modulators and has used a closed perfusion system maintained under constant pressure and environmentally controlled. An advantage of this technique is that rapid removal of free ligand upon reservoir exchange allows agonist dissociation to be performed in the absence of a saturating concentration of competitive orthosteric ligand. Specifically this study has directly quantified, at the single cell level, the kinetics of the fluorescent adenosine derivative, ABA-X-BY630 (Middleton et al., 2007), at the human A₁AR and A₃AR. Furthermore, the influence of the allosteric modulators, PD81,723 and VUF5455, on the kinetics of ABA-X-BY630 binding and function has been investigated at the human A₁AR and A₃AR in live cells.
MATERIALS AND METHODS

Materials

Fluo-4AM and pluronic acid were from Invitrogen (Oregon, USA), fetal calf serum was from PAA Laboratories (Pasching, Austria), ABA-X-BY630 was from CellAura Technologies Ltd (Nottingham, UK), L-glutamine and trypsin from Lonza (Verviers, Belgium), the ERK Surefire™ kits were from PerkinElmer Life Sciences. VUF 5455 was synthesized by B. Kellam (Centre for Biomolecular Sciences, School of Pharmacy, University of Nottingham, UK). All other chemicals were from Sigma-Aldrich Inc (St Louis, MO, USA).

Cell Culture

Chinese hamster ovary (CHO-K1) cell lines stably expressing either the human adenosine A₁ receptor (CHO–A₁; B\textsubscript{MAX}=3350±315 fmol/mg protein; Cordeaux et al., 2000) or the human adenosine A₃ receptor (CHO–A₃; B\textsubscript{MAX}=765±22 fmol/mg protein; Cordeaux et al., 2008) respectively, were maintained in Dulbecco’s modified Eagle’s medium (DMEM)/nutrient mix F12 (F-12) containing 10% fetal calf serum (FCS) and 2 mM L-glutamine at 37°C in a humidified incubator containing 5% CO₂. When confluent, cells were harvested (0.25% trypsin), centrifuged (1000\( \times \)g for 5 min) and the resulting pellet resuspended in DMEM-12 containing 10% FCS and 2 mM glutamine. Prior to assaying, cells were seeded in DMEM-F12 containing 10% FCS and 2 mM glutamine for at least 18 hours and grown to 80-100% confluence.

Calcium Mobilization Assay

CHO–A₁ and CHO–A₃ cells seeded into black-sided 96-well view plates were incubated in 100 µL HEPES-buffered saline (HBSS; 25 mM HEPES, 10 mM glucose, 146
mM NaCl, 5 mM KCl, 1 mM MgSO₄, 2 mM sodium pyruvate, 1.3 mM CaCl₂) containing 0.1% BSA, 2.5 mM probenecid, 0.5 mM Brilliant Black BN, 2.3 μM Fluo-4AM and 0.023% pluronic acid at 37°C for 1 hour. Plates were then loaded onto a fluidics plate reader (FlexStation, Molecular Devices, Sunnyvale, CA, USA) and fluorescence measured (excitation = 485 nm, emission = 520 nm) every 1.52 seconds for up to 200 seconds with the addition of HBSS in the absence or presence of ABA-X-BY630 at 15 seconds.

**ERK1/2 Phosphorylation Assay**

ERK1/2 phosphorylation was measured using the ERK Surefire™ kit (PerkinElmer). CHO-A₁ and CHO-A₃ cells seeded into 96-well clear plates were washed twice with PBS and maintained in 100 μL DMEM-F12 containing 2 mM glutamine (DMEM-F12/glutamine) for at least 4 hours before assaying. Cells were then exposed to ABA-X-BY630 for 5 minutes, after which stimulation was terminated by the removal of media and the addition of 40 μL of SureFire™ lysis buffer to each well. A 1:80:20:120 v/v dilution of AlphaScreen™ beads:lysate:activation buffer:reaction buffer in an 5.5 μL total volume was then transferred to a white opaque 384-well Proxiplate™ in the dark. This plate was then incubated in the dark at 37°C for 2 hours after which time the fluorescence signal was measured by an Envision plate reader (PerkinElmer) using standard AlphaScreen™ settings.

**Confocal Microscopy**

Live cell imaging of non-transfected CHO-K1 (CHO-NT), CHO-A₁ and CHO-A₃ cells seeded onto 32 mm circular coverslips was performed using a Zeiss LSM 510 laser scanning confocal microscope and a Zeiss Plan-Neofluar 40 x 1.3 NA oil-immersion objective. A 633 nm HeNe laser was used for the excitation of ABA-X-BY630 (a BODIPY630/650 conjugate) with emission being detected using a 650nm long-pass filter. A
custom made, temperature controlled perfusion system was utilized for drug delivery and removal. The perfusion system consisted of an imaging cell (a closed chamber with a total volume of 400 μL) coupled to six fluid reservoirs. A manually operated three-way valve system was employed to select the reservoir for use and a constant rate of flow maintained by means of an air pressure manifold. For the duration of each experiment, phase and fluorescence images were captured every 2 seconds. Within each field of view, a region of interest was drawn around the plasma membrane of 10 cells and the changes in fluorescence intensity determined over time (Zeiss AIM 4.2 Software).

Characterizing the association and dissociation kinetics of ABA-X-BY630 at the A1AR and A3AR.

Initial live cell kinetic experiments, characterizing the association and dissociation kinetics of ABA-X-BY630 (3 – 100 nM), involved 30 seconds of HBSS perfusion to obtain the basal fluorescence followed by 4 minutes exposure of cells to ABA-X-BY630 after which ABA-X-BY630 perfusion was terminated and replaced by HBSS-only perfusion. In each case, the temperature was maintained at 37°C, the flow rate was equal to, or greater than 5 mL/min and the pinhole diameter (1 Airy Unit; 1.1 μm optical slice), laser power and gain were kept constant within experiments using the same receptor subtype.

Characterizing the influence of the allosteric modulators, PD81,723 and VUF5455, on ABA-X-BY630 dissociation from the A1AR and A3AR respectively.

CHO-A1 and CHO-A3 cells were exposed to 30 nM ABA-X-BY630 alone for two minutes, after which CHO-A1 cells only were exposed to 30 nM ABA-X-BY630 in the absence and presence of 10 μM PD81,723 for an additional minute. Subsequent to association, ABA-X-BY630 dissociation from A1ARs and A3ARs was assessed by the
perfusion of HBSS in the absence or presence of 10 μM PD81,723 (CHO-A1) or 1 μM VUF5455 (CHO-A3). The configuration of the Zeiss 510 laser scanning confocal microscope was kept constant between experiments. Experiments investigating the influence of the competitive ligand, DPCPX, on ABA-X-BY630 dissociation from the A1AR involved two minute exposure of CHO-A1 cells to 100 nM ABA-X-BY630 alone, followed by perfusion of HBSS containing 100 nM DPCPX. The configuration of the Zeiss 510 laser scanning confocal microscope was kept constant between experiments.

Simultaneous investigation of the influence of PD81,723 and VUF5455 on the kinetics of ABA-X-BY630 binding and function.

CHO-A1 and CHO-A3 cells seeded onto 32mm circular coverslips were incubated in 1.5 mL Dulbecco’s modified Eagle’s medium (DMEM)/nutrient mix F12 (F-12) containing 10% fetal calf serum (FCS), 2 mM L-glutamine, 2.5 mM probenecid, 2.3 μM Fluo-4AM and 0.023% pluronic acid for 45-90 minutes at 37°C in a humidified incubator containing 5% CO2. Cells were then loaded onto the perfusion system and washed with HBSS perfusion. During the experiment, cells were pretreated for 1 minute with HBSS in the presence or absence of allosteric modulator, PD81,723 (1 μM, 10 μM) or VUF5455 (0.1 μM, 1 μM), after which they were exposed to 30 nM ABA-X-BY630 in the presence of the pretreatment condition for an additional minute. ABA-X-BY630 dissociation was then initiated by perfusion of the pretreatment condition alone. A 488nm argon laser was used for the excitation of Fluo-4 with emission being detected using a 505-550nm band-pass filter. The configuration of the Zeiss 510 laser scanning confocal microscope was kept constant between experiments. As above, ABA-X-BY630 binding was assessed by drawing a region of interest around the plasma membrane of 10 cells within each field of view. In contrast, given the oscillatory and non-synchronous calcium response, the complete field of view was used to
estimate the calcium mobilization mediated in the presence of ABA-X-BY630 (Zeiss AIM 4.2 Software).

Data Analysis

All data were fitted using Prism 5 (GraphPad Software, San Diego, CA).

Concentration-response curves for ABA-X-BY630 were fitted to the following equation:

\[
\text{Response} = \frac{E_{\text{MAX}} \times [\text{ABA-X-BY630}]}{[\text{ABA-X-BY630}]+[\text{EC}_{50}]}
\]  
(Equation 1)

where \(E_{\text{MAX}}\) is the maximal response and the \(\text{EC}_{50}\) is the molar concentration of ABA-X-BY630 required to generate 50% of \(E_{\text{MAX}}\). Association kinetic data were fit to the following mono-exponential association function:

\[
Y = Y_0 + (\text{Plateau} - Y_0)(1 - e^{-k_{\text{onobs}}t})
\]  
(Equation 2)

where \(Y_0\) represents the level of ABA-X-BY630 binding at time \(t\) equal to zero, plateau is the level of binding at infinite time and \(k_{\text{onobs}}\) is the rate of observed association. Binding of 3 nM and 10 nM ABA-X-BY630 showed a low level of non-specific binding (less than 10%) and as such association and dissociation kinetic data could be simultaneously fitted to Equation 2 and the following mono-exponential decay function:

\[
Y = (Y_0 - \text{Plateau}) \cdot e^{-k_{\text{off}}t} + \text{Plateau}
\]  
(Equation 3)

where \(Y_0\), \(t\) and the plateau are as previously defined and \(k_{\text{off}}\) is the rate of \([\text{ABA-X-BY630}]\) dissociation. Binding of 30 nM and 100 nM ABA-X-BY630 had a greater contribution of non-specific binding and as such was fitted individually to Equation 2 and the following two phase exponential decay:

\[
Y = \text{Plateau} + \text{Span}_{\text{fast}} \cdot e^{-k_{\text{off(fast)}}t} + \text{Span}_{\text{slow}} \cdot e^{-k_{\text{off(slow)}}t}
\]  
(Equation 4)
where plateau and \( t \) are as previously defined and \( \text{span}_{\text{fast}} \) and \( \text{span}_{\text{slow}} \) represent the proportion of \( Y_0 \) - plateau accounted for by the faster \( (k_{\text{off(fast)}}) \) and slower \( (k_{\text{off(slow)}}) \) rate of dissociation respectively. Within this analysis, \( k_{\text{fast}} \) was constrained to equal 3.81 min\(^{-1}\), the average rate of dissociation of ABA-X-BY630 from non-transfected CHO-K1 cells. The association rate \( (k_{\text{on}}) \) and negative logarithm of the equilibrium dissociation constant \( (pK_D) \) were estimated according to the following equations:

\[
k_{\text{on}} = \frac{k_{\text{onobs}} - k_{\text{off(slow)}}}{[ABA-X-BY630]} \quad \text{(Equation 5)}
\]

and

\[
pK_D = -\log \left( \frac{k_{\text{off(slow)}}}{k_{\text{on}}} \right) \quad \text{(Equation 6)}
\]

where \( k_{\text{onobs}}, k_{\text{off(slow)}} \) and \( k_{\text{on}} \) are as previously defined. Experiments investigating the influence of PD81,723 and VUF5455 on ABA-X-BY630 association and dissociation were expressed as specific binding and globally analyzed using the following equations:

Association:

\[
Y = \frac{B_{\text{MAX}} \times (3 \times 10^{-8})}{((3 \times 10^{-8}) + K_D)} \times \left( 1 - e^{-\left((3 \times 10^{-8}) \times k_{\text{on}} + k_{\text{off}}\right) \times t} \right) + NS \quad \text{(Equation 7)}
\]

Dissociation:

\[
Y = \frac{B_{\text{MAX}} \times (3 \times 10^{-8})}{((3 \times 10^{-8}) + K_D)} \times \left( 1 - e^{-\left((3 \times 10^{-8}) \times k_{\text{on}} + k_{\text{off}}\right) \times (t-1)} \times e^{-k_{\text{off}} \times (t-1)} \right) + NS \quad \text{(Equation 8)}
\]

where \( B_{\text{MAX}} \) represents the fluorescence intensity upon complete receptor occupation. \( B_{\text{MAX}} \) was shared across all experiments using the same receptor subtype. \( NS \) represents non-
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specific binding. $K_D$, $k_{on}$, $k_{off}$ and $t$ are as previously defined. Equilibrium dissociation constants and potency values were estimated as logarithms and all values are expressed as the mean ± the standard error of the mean (S.E.M.).

Statistical analysis

Statistical analysis involved one-way ANOVA or unpaired $t$ tests, where appropriate, with statistical significance reflecting $p < 0.05$ (Prism 5, GraphPad Software, San Diego, CA). When $p < 0.05$ the one-way ANOVA was followed by either a Tukey’s multiple comparison test or a Dunnett’s multiple comparison test, where appropriate.

RESULTS

The nature of the fluid exchange within the perfusion system

This study has used a closed perfusion system, consisting of an imaging cell coupled to six reservoirs (Figure 1a), in conjunction with a Zeiss LSM 510 laser scanning confocal microscope to directly visualize and quantify fluorescent ligand association and dissociation kinetics at the single cell level. The perfusion system was environmentally controlled, maintained at the physiological temperature of 37°C, and kept at constant pressure, promoting laminar flow and preventing loss of focus. Another important aspect of this system is the use of ‘infinite dilution’ to assess agonist dissociation kinetics (Christopoulos et al., 1997; Lee and el-Fakahany, 1991). The method of ‘infinite dilution’ requires dissociated fluorescent ligand to be rapidly removed such that the level of ligand re-association is negligible. To investigate the nature of the fluid transfer within the perfusion system, CHO-K1 cells were exposed to the dye, Brilliant Black BN. The introduction of 50 μM Brilliant Black BN within the imaging cell at a flow rate of 5 mL/min caused a rapid decrease in the intensity of the phase image which quickly recovered upon the removal of the dye (Figure
1b). Fitting an exponential function to the onset and removal of Brilliant Black BN, estimated a half time of 1.6 and 1.4 seconds respectively suggesting that it was possible to obtain rapid fluid transfer within the imaging cell.

To further confirm the lack of ligand re-association during the dissociation phase, the binding kinetics of the fluorescent adenosine derivative, ABA-X-BY630, at CHO-A<sub>3</sub> cells were investigated at differing flow rates. The association and dissociation rates of 100 nM ABA-X-BY630 at the A<sub>3</sub>AR were similar at flow rates of 3 – 5 mL/min, 6 – 10 mL/min, 11 – 15 mL/min or 16 – 20 mL/min, which reflect 7.5 – 12.5, 15 – 25, 27.5 – 37.5 or 40 – 50 complete fluid exchanges within the imaging cell per minute (Figure 1c). As such, association appears to be initiated by the exposure of cells to the desired concentration of free ligand, which in turn can be rapidly removed upon the initiation of dissociation. Taken together, these results suggest that the fluid exchange within the perfusion system involves a sharp change in the concentration gradient of free ligand upon reservoir changeover and as such the method of ‘infinite dilution’ can be used to assess fluorescent ligand dissociation. All subsequent experiments use a flow rate equal to or greater than 5 mL/min.

**Investigating the functional activity of ABA-X-BY630 at the A<sub>1</sub>AR and A<sub>3</sub>AR**

The agonist activity of the adenosine derivative, ABA-X-BY630, at the A<sub>1</sub>AR and/or A<sub>3</sub>AR was confirmed in both calcium mobilization and extracellular signal-regulated kinase 1 and 2 (ERK1/2) phosphorylation assays. Both A<sub>1</sub>ARs and A<sub>3</sub>ARs preferentially couple to G<sub>o</sub> type G-proteins (Fredholm et al., 2001). As such, upon stimulation, these receptors mediate inhibition of adenylyl cyclase through the α subunit of the G-protein. In addition, the βγ subunits of the G-protein can stimulate intracellular calcium mobilization and ERK1/2 phosphorylation through direct interactions with phospholipase C (Rhee, 2001). ABA-X-
BY630 mediated a concentration-dependent increase in intracellular calcium mobilization and ERK1/2 phosphorylation at both the A₁AR and A₃AR (Figure 2a and 3a). When compared to the response to a non-selective full agonist, 5′-(N-ethyl carboxamido)adenosine (NECA), ABA-X-BY630 mediated a partial stimulation of calcium mobilization (Eₘₐₓ of 59 ± 3% and 52 ± 7% respectively) but a level of ERK1/2 phosphorylation that was not significantly different from that of NECA at both the A₁AR and A₃AR. The potency (pEC₅₀) of ABA-X-BY630 at mediating intracellular calcium mobilization and ERK1/2 phosphorylation was 6.1 ± 0.1 and 7.7 ± 0.2 respectively at the A₁AR and 5.9 ± 0.2 and 7.3 ± 0.2 respectively at the A₃AR.

**Characterization of agonist kinetics at the A₁AR and A₃AR**

Association and dissociation kinetics of ABA-X-BY630 were assessed at both the A₁AR and A₃AR. For the purpose of direct comparison, the configuration of the Zeiss 510 laser scanning confocal microscope and agonist exposure time (4 minutes) were kept constant between the different concentrations of ABA-X-BY630. Confocal fluorescence and phase images were obtained at two second intervals for the duration of the experiment. Discrete binding of 3 nM ABA-X-BY630 to the cell membrane could only be detected for CHO-A₃ cells, whereas 10, 30 and 100 nM ABA-X-BY630 was observed at both CHO-A₁ and CHO-A₃ cells. As would be expected, the fluorescence intensity of ABA-X-BY630 detected at the cell membrane increased with ligand concentration (Figure 2c and 3c).

Repeating association and dissociation experiments using non-transfected CHO (CHO-NT) cells allowed quantification of the non-specific binding of ABA-X-BY630. For the purpose of comparison, non-specific association and dissociation kinetics were defined according to the confocal settings used for both CHO-A₁ and CHO-A₃ cells. Importantly, the
fluorescence intensity of non-specific binding was considerably lower than that observed for the corresponding concentration of ABA-X-BY630 in CHO-A1 and CHO-A3 cells. Furthermore for both sets of confocal configurations, membrane localized non-specific binding could only be accurately quantified at 30 and 100 nM ABA-X-BY630 (Figure 2d and 3d). The observed association and dissociation of non-specific binding was rapid, suggesting ABA-X-BY630 has very low affinity for, and therefore was unlikely to become sequestered within, the plasma membrane. The observed association rate (konobs) and dissociation kinetics (koff) for corresponding ABA-X-BY630 concentrations using the CHO-A1 and CHO-A3 configurations did not cause a significant difference (unpaired t test) in the non-specific binding kinetics and as such are represented as a combined value (Table 1). In addition, no significant difference (unpaired t test) was observed between the dissociation rate of 30 and 100 nM from CHO-NT cells.

Normalizing the binding of 3 (CHO-A3 only), 10, 30 and 100 nM ABA-X-BY630 at CHO-A1 (Figure 2e & 2f) and CHO-A3 (Figure 3e & 3f) cells clearly demonstrates the concentration-dependent nature of the observed association rate and the concentration-independent nature of the dissociation kinetics. At both A1ARs and A3ARs the observed association for each concentration of ABA-X-BY630 preferentially fit to a mono-exponential curve. The dissociation of 3 and 10 nM ABA-X-BY630 preferentially fit to a single phase exponential decay. In contrast, two components could be detected for the dissociation of 30 and 100 nM ABA-X-BY630. In each case, the proportion and rate of the rapidly dissociating component was similar to that expected for, and therefore was assumed to represent, non-specific binding. As such, the fast component of the biphasic decay was constrained to equal the average dissociation rate estimated for non-specific binding (koff(fast) = 3.81 min⁻¹). At both A1ARs and A3ARs, one-way ANOVA analysis suggested that there was a significant
difference between the $k_{\text{onobs}}$, but not the $k_{\text{off}}$ estimates for the different concentrations of ABA-X-BY630 (Table 1). The observed association rate ($k_{\text{onobs}}$) and the slow component of the biphasic decay ($k_{\text{off(slow)}}$) were used to estimate the association rate ($k_{\text{on}}$) of ABA-X-BY630 at the A1AR and A3AR (Equation 5). Subsequently, the association ($k_{\text{on}}$) and dissociation ($k_{\text{off}}$) rates were used to estimate the affinity of ABA-X-BY630 at both receptors (Equation 6). Analysis of these data according to one-way ANOVA suggested that there was no significant difference between the association rate constants ($k_{\text{on}}$) or equilibrium dissociation constants ($pK_D$) calculated for the different concentrations of ABA-X-BY630 (Table 1).

**Single cell analysis of agonist binding parameters**

Single cell analysis of agonist binding kinetics was performed for 3, 10, 30 and 100 nM ABA-X-BY630 at A3ARs (Figure 4). As for the grouped analysis, ABA-X-BY630 kinetics were analyzed according to a monoexponential association followed by a monophasic decay for 3 and 10 nM and a biphasic decay with $k_{\text{off(fast)}}$ constrained to equal $3.81 \text{ min}^{-1}$ for 30 and 100 nM. Similar to the grouped analysis, results of the single cell analysis suggested that the dominant influence of fluorescent ligand concentration was on the $k_{\text{onobs}}$ with a coefficient of determination ($r^2$) for a linear trend of 0.488. When compared to $k_{\text{onobs}}$, there was less correlation between ligand concentration and the estimated $k_{\text{off(slow)}}$, $k_{\text{on}}$ and $pK_D$ from the single cell analysis which had $r^2$ values of 0.13, 0.12 and 0.01 respectively.

**Investigating the influence of allosteric modulators on agonist dissociation kinetics in whole cells**

Ligands competing for the same site on a monomeric receptor should not influence the dissociation kinetics of one another (Figure 5a). This is clearly shown in Figure 5b where
the addition of the competitive ligand, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX; 100 nM), during the dissociation phase does not influence the dissociation of ABA-X-BY630 (100 nM). In contrast, if ligands bind simultaneously to two interacting sites, such as in the case of allostery, cooperative interactions can alter ligand dissociation kinetics (Christopoulos and Kenakin, 2002; May et al., 2007b). To date, the majority of studies investigating the influence of allosteric modulators on GPCRs use a saturating concentration of unlabelled orthosteric ligand in the absence or presence of allosteric modulator to bind any unoccupied orthosteric binding sites and as such inhibit the re-association of dissociated labeled orthosteric ligand (Christopoulos et al., 1997; Lee and el-Fakahany, 1991). In contrast, this system has used the method of ‘infinite dilution’ to assess the dissociation rate of the fluorescent agonist in the absence and presence of allosteric modulator. This method eliminates a number of the issues that can arise when using additional orthosteric ligands and also allows agonist kinetics to be assessed within whole cells. The dissociation of 30 nM ABA-X-BY630 from CHO-A1 cells was significantly slower in the presence (k_{off}=0.51 \pm 0.03 \text{ min}^{-1}; n=6) as compared to the absence (k_{off}=1.10 \pm 0.06 \text{ min}^{-1}; n=14) of 10 \mu M PD81,723 (Figure 5c). Supporting the population data, single cell analysis shows a clear decrease in the dissociation rate of 30 nM ABA-X-BY630 at the A1AR in presence of 10 \mu M PD81,723 (Figure 5d). In contrast, the dissociation of 30 nM ABA-X-BY630 from CHO-A3 cells was significantly enhanced in the presence (k_{off}=3.15 \pm 0.12 \text{ min}^{-1}; n=4) as compared to the absence (k_{off}=0.68 \pm 0.18 \text{ min}^{-1}; n=4) of 1 \mu M VUF5455 (Figure 5e). This enhanced dissociation rate of 30 nM ABA-X-BY630 from the A3AR in the presence of 1 \mu M VUF5455 can also be observed at the single cell level (Figure 5f).

**Investigating the influence of allosteric modulators on the kinetics of ABA-X-BY630 binding and function**
The influence of PD81,723 (1 \(\mu M\) and 10 \(\mu M\)) and VUF5455 (0.1 \(\mu M\) and 1 \(\mu M\)) on the kinetics of ABA-X-BY630 (30 nM) association and dissociation was investigated simultaneously with their effect on receptor-mediated calcium mobilization. At the A1AR, the presence of 1 \(\mu M\) PD81,723 had no significant effect on the binding of 30 nM ABA-X-BY630 whereas 10 \(\mu M\) PD81,723 caused an approximate 2-fold increase in the level of 30 nM ABA-X-BY630 specific binding (Figure 6a). Global analysis of association and dissociation curves suggested that the increase in 30 nM ABA-X-BY630 binding in the presence of 10 \(\mu M\) PD81,723 was due a significant increase in the association (\(k_{on}\)) and decrease in the dissociation (\(k_{off}\)) rate constants for ABA-X-BY630 at the A1AR respectively. This in turn resulted in a significant increase in the apparent affinity of ABA-X-BY630 for the A1AR in the presence of 10 \(\mu M\) PD81,723 (Table 2). The significant decrease in the rate of 30 nM ABA-X-BY630 dissociation (\(k_{off}\)) in the presence of 10 \(\mu M\) PD81,723 can be clearly observed when the data was normalized (Figure 6d). In contrast, there is no significant difference between the observed rate of association (\(k_{onobs}\)) for ABA-X-BY630 at the A1AR in the absence and presence of 1 \(\mu M\) or 10 \(\mu M\) PD81,723 (Figure 6c). This could result from the opposing effects an increased association rate (\(k_{on}\)) and a decreased dissociation rate (\(k_{off}\)) has on the observed rate of association (\(k_{onobs}\)). At the A1AR, 1 \(\mu M\) VUF5455 mediated a significant decrease in the dissociation rate (\(k_{off}\)) of 30 nM ABA-X-BY630, suggesting that VUF5455 has an allosteric mechanism of action at the A1AR (Figure 6h; Table 2). However, despite decreasing the dissociation rate, a concurrent decrease in the association rate constant (\(k_{on}\)) in the presence of 1 \(\mu M\) VUF5455 caused a decrease in the level of 30 nM ABA-X-BY630 specific binding at the A1AR (Table 2; Figure 6e). There was no significant difference between the observed association rate (\(k_{onobs}\)) of 30 nM ABA-X-BY630 at the A1AR in the absence or presence of 0.1 \(\mu M\) or 1 \(\mu M\) VUF5455 (Figure 6g).
At the A3AR, PD81,723 mediated a concentration-dependent decrease in the binding of 30 nM ABA-X-BY630, an increase in the dissociation rate (k_{off}) and a decrease in the association rate (k_{on}) of 30 nM ABA-X-BY630 (Figure 7a; Table 2). The combined changes in ABA-X-BY630 kinetics resulted in a concentration-dependent decrease in the apparent affinity of ABA-X-BY630 for the A3AR in the presence of PD81,723 (Table 2). Normalized data clearly illustrates the significant increase in the dissociation rate (k_{off}) of 30 nM ABA-X-BY630 at the A3AR in the presence of 1 \mu M and 10 \mu M PD81,723 (Figure 7d). There was no significant difference between the observed association rate (k_{onobs}) of 30 nM ABA-X-BY630 in the absence and presence of 1 \mu M or 10 \mu M PD81,723 (Figure 7c). As above, this could be due to the opposing influence of PD81,723 on the rate constants for association and dissociation manifesting as opposing effects on the observed rate of association of 30 nM ABA-X-BY630 at the A3AR (Figure 7g).

At the A3AR, the presence VUF5455 mediated a concentration-dependent decrease in binding of 30 nM ABA-X-BY630 such that in the presence of 1 \mu M VUF5455, the level of 30 nM ABA-X-BY630 binding was indistinguishable from non-specific binding (Figure 7e). The decrease in 30 nM ABA-X-BY630 binding at the A3AR in the presence of 0.1 \mu M VUF5455 was due to a decrease in the rate of association (k_{on}) and an increase in the rate of dissociation (k_{off}) (Table 2). Rate constants in the presence of 1 \mu M VUF5455 could not be determined due to the negligible level of 30 nM ABA-X-BY630 specific binding under these conditions. Normalized data clearly illustrates the increased dissociation rate (k_{off}) of ABA-X-BY630 at the A3AR in the presence of 0.1 \mu M VUF5455 (Figure 7h); in contrast there was no significant difference between the observed association rate (k_{onobs}) of 30 nM ABA-X-BY630 in the absence and presence of 0.1 \mu M VUF5455 at the A3AR (Figure 7g).
Calcium mobilization mediated through both the A1AR and the A3AR in the presence of 30 nM ABA-X-BY630 was investigated simultaneously to binding. In CHO-A1 and CHO-A3 cells, 30 nM ABA-X-BY630 mediated a non-synchronous, oscillatory calcium response. On average in CHO-A1 cells, in the presence of 30 nM ABA-X-BY630 calcium mobilization was initiated at approximately 10% receptor occupancy whereas the peak response occurred at 25% receptor occupancy (Figure 6b). On average in CHO-A3 cells, calcium mobilization was initiated at approximately 15% receptor occupancy whereas the peak response to 30 nM ABA-X-BY630 occurred at 65% receptor occupancy (Figure 7b). The presence of 1 μM or 10 μM PD81,723 had no significant effect on the calcium response mediated by 30 nM ABA-X-BY630 at the A1AR (Figure 6b). In contrast at the A3AR, while 1 μM PD81,723 had no significant effect, the presence of 10 μM PD81,723 reduced the peak calcium response mediated by 30 nM ABA-X-BY630 by greater than 90% (Figure 7b). At the A3AR, calcium mobilization in response to 30 nM ABA-X-BY630 was decreased by approximately 90% in the presence of 0.1 μM VUF5455 and could not be detected in the presence of 1 μM VUF5455 (Figure 7f). In contrast at the A1AR, peak calcium mobilization in response to 30 nM ABA-X-BY630 was not significantly influenced by 0.1 μM or 1 μM VUF5455 (Figure 6f).

DISCUSSION

Association and dissociation kinetics of ligand binding represents a sensitive method to detect changes in receptor conformation. This study has provided the first direct assessment of the kinetics of a GPCR small molecule agonist in the absence and presence of allosteric modulators in single living cells. As would be predicted from simple mass action, at both A1ARs and A3ARs the observed association rate (k_{onobs}) of ABA-X-BY630 binding
was concentration-dependent. In contrast, the rate constants for association ($k_{\text{on}}$) and dissociation ($k_{\text{off(slow)}}$) as well as the equilibrium dissociation constant ($pK_D$), were concentration-independent. Analysis of intact cell association and dissociation kinetics of ABA-X-BY630 suggest that both PD81,723 and VUF5455 have an allosteric mechanism of action at the $A_1$AR and the $A_3$AR. At the $A_1$AR, PD81,723 and VUF5455 significantly decrease the dissociation rate of ABA-X-BY630. In contrast at the $A_3$AR, VUF5455 and PD81,723 significantly enhance the dissociation rate of ABA-X-BY630.

Numerous advantages have been gained through assessing agonist kinetics using a pressurized, closed perfusion system in conjunction with confocal microscopy. For example, the maintenance of a constant pressure combined with the closed nature of the viewing cell promotes laminar flow with a constant flow rate and prevents loss of focus. These features also generate a sharp concentration gradient between fluid exchanges, allowing fluorescent ligand dissociation to be quantified in the absence of saturating concentrations of additional ligands. Imaging ligand kinetics using confocal microscopy can generate virtually continuous association and dissociation profiles on a single population of cells; removing previous requirements to quantify ligand kinetics using endpoint assays and allowing the dynamics of ligand kinetics to be quantified at physiologically relevant temperatures. Imaging also allows discrimination between plasma membrane and intracellular ligand binding. An additional advantage of this method is that it is readily amenable to the study of endogenously expressed receptors in intact primary cells.

The average $pK_D$ of ABA-X-BY630, at the $A_1$AR and $A_3$AR, was 7.3 and 7.9 respectively. The $pK_D$ of adenosine for the high and low affinity states at the $A_1$AR has previously been estimated as 7.9 and 5.1 respectively (Cohen et al., 1996a). As such it appears the low concentrations of ABA-X-BY630 used within this study predominantly
detect high affinity receptor states. This finding is consistent with those from a previous study using an alternative single cell technique, fluorescence correlation spectroscopy (FCS), which assessed the ability of NECA to inhibit the binding of a low concentration (2.5 nM) of the fluorescent agonist, ABEA-X-BY630 (Cordeaux et al., 2008). The affinity ($pK_D$) of NECA for the human $A_3$AR in intact CHO-$A_3$ cells was approximately 8.5. For non-peptide GPCRs, low nanomolar equilibrium dissociation constant estimates are likely to represent agonist binding to active receptor conformations. According to the cubic ternary complex model, the active receptor conformation can be both G protein-coupled or G protein-uncoupled (Kenakin, 2002). In living cells, the presence of GTP causes rapid dissociation of the active ligand/receptor/G protein ternary complex. As such, we speculate that these studies predominantly detect active G protein-uncoupled receptor conformations. This suggestion is supported by the difference between agonist kinetics estimated from live cells in the current study and those from previous studies in membrane homogenates. At both the $A_1$AR and $A_3$AR, agonist kinetics in intact cells were at least 10 times faster than previous estimates of agonist kinetics from membrane homogenates (Cohen et al., 1996b; Gao et al., 2001).

In addition to orthosteric agonists and antagonists, GPCRs can also interact with intracellular proteins, transmembrane receptors and allosteric small molecules. Cooperative interactions between two interacting binding sites are generally manifested as a change in ligand binding kinetics (May et al., 2007b). However, due to the methodological limitations mentioned, allosteric modulation of agonist binding kinetics at non-peptide GPCRs have not been investigated in whole cells. Allosteric binding sites on $A_1$ARs and $A_3$ARs represent an attractive therapeutic target for amplifying the tissue protective actions of endogenous adenosine in conditions such as ischemia, chronic neuropathic pain and inflammation (Cohen
and Downey, 2008; Jacobson and Gao, 2006). A number of compounds, such as PD81,723 and VUF5455, have been identified that can retard the dissociation of radiolabeled agonists in the presence of a high concentration of unlabelled orthosteric antagonist from cell membrane homogenates expressing the A1AR and A3AR (Bruns and Fergus, 1990; Gao et al., 2001). The pre-requisite for the presence of a saturating concentration of unlabelled orthosteric antagonist in these membrane radioligand binding assays (in order to prevent rebinding of the radioligand) means that any observed effect of the allosteric ligand can be influenced by the presence of the non-radioactive orthosteric ligand and assumptions have to be made about the orthosteric nature of the competing ligand. In contrast, the method described within this manuscript assesses the dissociation of the labeled ligand under conditions of ‘infinite dilution’ and as such does not require such underlying assumptions. Both PD81,723 and VUF5455 were able to modulate ABA-X-BY630 dissociation kinetics in single living cells. Similar to previous findings, PD81,723 (10 μM) significantly retarded the dissociation of fluorescent agonist from the A1AR. In contrast to decreasing the rate of agonist dissociation, this study has found that VUF5455 significantly enhanced the dissociation of ABA-X-BY630 from the A3AR. A number of possibilities could account for the observed discrepancy. For example, allosteric modulators can be highly probe-dependent and as such the difference may result from the inconsistency in the agonist probe used (May et al., 2007b). Alternatively, the difference could reflect a more complex receptor arrangement than a non-interacting monomer or a multisite action of the non-radioactive “orthosteric” ligand employed in the radioligand membrane binding assay, that is, both an orthosteric and an allosteric mechanism of action.

To further investigate the mechanism of allosteric modulation, the influence of the PD81,723 and VUF5455 on the association rate constant (k_on), affinity and function of ABA-
X-BY630 was investigated at both the A₁AR and the A₃AR. An advantage of the perfusion system in conjunction with a confocal microscope is that fluorescent ligand binding can be performed simultaneously to function when using a fluorescence readout such as the calcium sensitive fluorescent indicator, Fluo-4. At the A₁AR, in addition to decreasing the dissociation rate, PD81,723 enhanced the association rate of ABA-X-BY630. Combined, this caused an approximate 4-fold increase in the apparent affinity of 30 nM ABA-X-BY630 in the presence of 10 µM PD81,723. However, despite causing an approximately 2-fold increase in the specific binding of 30 nM ABA-X-BY630, 10 µM PD81,723 had no significant effect on calcium mobilization mediated by 30 nM ABA-X-BY630 at the A₁AR. This may be because the non-synchronous and oscillatory calcium response causes small changes in the functional response to remain undetected, or alternatively although PD81,723 enhances ABA-X-BY630 affinity at the A₁AR, it may inhibit efficacy. PD81,723 was also found to have a previously unreported allosteric mechanism of action at the A₃AR. At the A₃AR, PD81,723 decreased the affinity of ABA-X-BY630 though a decrease in the association and an increase in the dissociation rate constant. As would be expected, the approximately 2-fold decrease in ABA-X-BY630 binding in the presence of 10 µM PD81,723 resulted in a significant decrease in the calcium mobilization mediated by 30 nM ABA-X-BY630 through the A₃AR. At the A₃AR, VUF5455 also acted as an allosteric inhibitor of ABA-X-BY630, increasing and decreasing the dissociation and association rate constants for ABA-X-BY630 respectively. The decrease in 30 nM ABA-X-BY630 binding at the A₃AR in the presence of 0.1 µM and 1 µM VUF5455 was coupled with a significant decrease in the calcium response mediated by 30 nM ABA-X-BY630 at the A₃AR. The current kinetic studies also suggest that VUF5455 may have an allosteric mechanism of action at the A₁AR. Although VUF5455 had no significant effect on the calcium
mobilization mediated by 30 nM ABA-X-BY630 through the A₁AR, 1 μM VUF5455 significantly decreased 30 nM ABA-X-BY630 dissociation and caused an approximately 2-fold decrease in binding.

This study has directly quantified the binding kinetics of the fluorescent adenosine derivative, ABA-X-BY630, in the absence and presence of allosteric modulators in live single cells. The allosteric modulators PD81,723 and VUF5455 slowed agonist dissociation at the A₁AR whereas at the A₃AR, both PD81,712 and VUF5455 significantly enhanced in ABA-X-BY630 dissociation. The potential therapeutic application for this class of allosteric modulators involves enhancing the binding of endogenous adenosine under conditions of metabolic stress. Therefore, the ability to observe the influence of allosteric modulators on the kinetics binding and function of a fluorescent derivative of the endogenous agonist in live cells in the absence of additional competitive ligands represents a significant advantage of this system over traditional methods and as such should significantly advance the understanding of orthosteric and allosteric ligand pharmacology at GPCRs.
ACKNOWLEDGMENTS

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REFERENCES


**FOOTNOTES**

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LEGENDS FOR FIGURES

Figure 1. The nature of fluid transfer upon reservoir exchange within the perfusion system. (a) A schematic of the perfusion system setup. (b) The addition of 50 μM Brilliant Black BN within the viewing cell caused a rapid decrease in the phase image intensity, which was readily reversed upon perfusion of HBSS only. (c) The influence of different flow rates, 3-5 mL/min (●), 6-10 mL/min (○), 11-15 mL/min (●) and 16-20 mL/min (○), on the association (0-2 minutes) and dissociation (2-5 minutes) kinetics of 100 nM ABA-X-BY630 at CHO-A3 cells. Data represent the mean ± the standard error of the mean (S.E.M.) from 3-5 separate experiments where each replicate reflects the fluorescence intensity from the plasma membrane of 10 cells.

Figure 2. Characterization of the binding and functional properties of ABA-X-BY630 at the human adenosine A1 receptor. (a) ABA-X-BY630 mediated a robust, concentration dependent, increase in intracellular calcium mobilization (○) and ERK1/2 phosphorylation (●) in CHO-A1 cells. Data are expressed as a percentage of the response mediated by 1 μM NECA and represent the mean ± S.E.M. from four experiments. (b) A confocal image showing discrete membrane binding of 30 nM ABA-X-BY630 at CHO-A1 cells. Association and dissociation kinetics of 10 (●), 30 (○), 100 (●) nM ABA-X-BY630 at CHO-A1 (c) and CHO-NT (d) cells. Normalized association (e) and dissociation (f) kinetics of 10 (●), 30 (○), 100 (●) nM ABA-X-BY630 at CHO-A1 cells. For the purpose of direct comparison, confocal configurations remained constant for both total and non-specific binding. Confocal fluorescence and phase images were obtained at two second intervals for the duration of the experiment. Data represent the mean ± S.E.M. from 3-6 separate experiments where each replicate reflects the fluorescence intensity from the plasma membrane of 10 cells.
Figure 3. Characterization of the binding and functional properties of ABA-X-BY630 at the human adenosine A3 receptor. (a) ABA-X-BY630 mediated a robust, concentration dependent, increase in intracellular calcium mobilization (○) and ERK1/2 phosphorylation (●) in CHO-A3 cells. Data are expressed as a percentage of the response mediated by 1 μM NECA and represent the mean ± S.E.M. from four experiments. (b) A confocal image showing discrete membrane binding of 30 nM ABA-X-BY630 at CHO-A3 cells. Association and dissociation kinetics of 3 (○), 10 (●), 30 (○), 100 (●) nM ABA-X-BY630 at CHO-A3 (c) and CHO-NT (d) cells. Normalized association (e) and dissociation (f) kinetics of 3 (○), 10 (●), 30 (○), 100 (●) nM ABA-X-BY630 at CHO-A3 cells. For the purpose of direct comparison, confocal configurations remained constant for both total and non-specific binding. Confocal fluorescence and phase images were obtained at two second intervals for the duration of the experiment. Data represent the mean ± S.E.M. from 4-8 separate experiments where each replicate reflects the fluorescence intensity from the plasma membrane of 10 cells.

Figure 4. Single cell analysis of the kinetic and equilibrium binding parameters of ABA-X-BY630 at the human adenosine A3 receptor. The dominant influence of ABA-X-BY630 concentration was on the observed association rate (a) as opposed the dissociation rate constant (b), association rate constant (c) or the equilibrium dissociation constant (d). The mean ± S.E.M. is shown for 60-80 replicates which are plotted as individual data points and represent the parameter estimates from single cells from 6-8 experiments.

Figure 5. Allosteric modulation of agonist dissociation in live cells. (a) A schematic illustrating the topographically distinct nature of allosteric and orthosteric receptor sites. (b) The dissociation of 100 nM ABA-X-BY630 in the absence (●; n=15) and presence of 100
nM DPCPX (●; n=2; data represents the mean only) from CHO-A1 cells. (e) The dissociation of 30 nM ABA-X-BY630 in the absence (●; n=14) and presence of 10 µM PD81,723 (●; n=6) from CHO-A1 cells. (d) Single cell analysis of the influence of 10 µM PD81,723 on the dissociation rate of 30 nM ABA-X-BY630 from CHO-A1 cells. The mean ± S.E.M. is shown for 120 replicates in the absence of 10 µM PD81,723 and 60 replicates in the presence of 10 µM PD81,723 which are plotted as individual data points and represent the rate of ABA-X-BY630 dissociation from single cells. (e) The dissociation of 30 nM ABA-X-BY630 in the absence (●; n=4) and presence of 1 µM VUF5455 (●; n=4) from CHO-A3 cells. Unless otherwise stated, data represent the mean ± S.E.M. where each replicate reflects the fluorescence intensity from the plasma membrane of 10 cells. (f) Single cell analysis of the influence of 1 µM VUF5455 on the dissociation rate of 30 nM ABA-X-BY630 from CHO-A3 cells. The mean ± S.E.M. is shown for 30 replicates in the absence of 1 µM VUF5455 and 40 replicates in the presence of 1 µM VUF5455 which are plotted as individual data points and represent the rate of ABA-X-BY630 dissociation from single cells.

**Figure 6.** The influence of PD81,723 and VUF5455 on the binding and function of 30 nM ABA-X-BY630 at the human adenosine A1 receptor. (a) The association and dissociation kinetics of 30 nM ABA-X-BY630 in the absence (●; n=12) and presence of 1 µM (○; n=10) and 10 µM (●; n=6) PD81,723 at CHO-A1 cells. (b) The intracellular calcium mobilization mediated by 30 nM ABA-X-BY630 in the absence (●; n=12) and presence of 1 µM (○; n=10) and 10 µM (●; n=6) PD81,723 at CHO-A1 cells. (c) Normalised association kinetics of 30 nM ABA-X-BY630 in the absence (●; n=12) and presence of 1 µM (○; n=10) and 10 µM (●; n=6) PD81,723 at CHO-A1 cells. (d) Normalised dissociation kinetics of 30 nM ABA-X-BY630 in the absence (●; n=12) and presence of 1 µM (○; n=10) and 10 µM (●;
n=6) PD81,723 at CHO-A1 cells. (e) The association and dissociation kinetics of 30 nM ABA-X-BY630 in the absence (●; n=12) and presence of 0.1 μM (○; n=3) and 1 μM (●; n=5) VUF5455 at CHO-A1 cells. (f) The intracellular calcium mobilization mediated by 30 nM ABA-X-BY630 in the absence (●; n=12) and presence of 0.1 μM (○; n=3) and 1 μM (●; n=5) VUF5455 at CHO-A1 cells. (g) Normalised association kinetics of 30 nM ABA-X-BY630 in the absence (●; n=12) and presence of 0.1 μM (○; n=3) and 1 μM (●; n=5) VUF5455 at CHO-A1 cells. (h) Normalised dissociation kinetics of 30 nM ABA-X-BY630 in the absence (●; n=12) and presence of 0.1 μM (○; n=3) and 1 μM (●; n=5) VUF5455 at CHO-A1 cells.

Figure 7. The influence of PD81,723 and VUF5455 on the binding and function of 30 nM ABA-X-BY630 at the human adenosine A3 receptor. (a) The association and dissociation kinetics of 30 nM ABA-X-BY630 in the absence (●; n=14) and presence of 1 μM (○; n=9) and 10 μM (●; n=6) PD81,723 at CHO-A3 cells. (b) The intracellular calcium mobilization mediated by 30 nM ABA-X-BY630 in the absence (●; n=14) and presence of 1 μM (○; n=9) and 10 μM (●; n=6) PD81,723 at CHO-A3 cells. (c) Normalised association kinetics of 30 nM ABA-X-BY630 in the absence (●; n=14) and presence of 1 μM (○; n=9) and 10 μM (●; n=6) PD81,723 at CHO-A3 cells. (d) Normalised dissociation kinetics of 30 nM ABA-X-BY630 in the absence (●; n=14) and presence of 1 μM (○; n=9) and 10 μM (●; n=6) PD81,723 at CHO-A3 cells. (e) The association and dissociation kinetics of 30 nM ABA-X-BY630 in the absence (●; n=14) and presence of 0.1 μM (○; n=7) and 1 μM (●; n=6) VUF5455 at CHO-A3 cells. (f) The intracellular calcium mobilization mediated by 30 nM ABA-X-BY630 in the absence (●; n=14) and presence of 0.1 μM (○; n=7) and 1 μM (●; n=6) VUF5455 at CHO-A3 cells. (g) Normalised association kinetics of 30 nM ABA-X-
BY630 in the absence (●; n=14) and presence of 0.1 μM (○; n=7) and 1 μM (●; n=6) VUF5455 at CHO-A3 cells. (h) Normalised dissociation kinetics of 30 nM ABA-X-BY630 in the absence (●; n=14) and presence of 0.1 μM (○; n=7) and 1 μM (●; n=6) VUF5455 at CHO-A3 cells.
Table 1: Kinetic and equilibrium parameters for ABA-X-BY630 at non-transfected CHO-K1 (CHO-NT) cells or CHO cells stably transfected with the A1AR or the A3AR (CHO-A1 and CHO-A3 cells respectively). Data were expressed as the mean ± the standard error of the mean of the stated number of replicates (n) where each n represents the average of 10 cells within a single experiment.

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<td>7</td>
</tr>
<tr>
<td>100</td>
<td>2.53 ± 0.26*$^a$</td>
<td>3.81</td>
<td>0.43 ± 0.11</td>
<td>2.10 ± 0.26</td>
<td>7.73 ± 0.14</td>
<td>6</td>
</tr>
</tbody>
</table>

N/A: not applicable.

** Statistically different ($p < 0.01$) from the corresponding $^a$10 nM ABA-X-BY630 value or $^b$3 nM ABA-X-BY630 value, as determined by one-way ANOVA followed by a Tukey’s multiple comparisons test.
Table 2: Global analysis of ABA-X-BY630 (30 nM) kinetic and equilibrium binding parameters in the absence and presence of PD81,723 and VUF5455 at the A1AR and A3AR. Data are expressed as the mean ± the standard error of the mean of the stated number of replicates (n) where each n represents the average of 10 cells within a single experiment.

<table>
<thead>
<tr>
<th></th>
<th>ABA-X-BY630</th>
<th>kon (×10^7 M⁻¹min⁻¹)</th>
<th>koff (min⁻¹)</th>
<th>pK_D(APPELLANT) (M⁻¹)</th>
<th>B_MAX (Fluorescence Intensity)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CHO-A1</strong></td>
<td>Control</td>
<td>2.62 ± 0.34</td>
<td>1.95 ± 0.09</td>
<td>7.13 ± 0.06</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+ 1 μM PD81,723</td>
<td>2.88 ± 0.39</td>
<td>1.72 ± 0.07</td>
<td>7.23 ± 0.06</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+ 10 μM PD81,723</td>
<td>4.17 ± 0.65*</td>
<td>0.80 ± 0.03*</td>
<td>7.72 ± 0.07*</td>
<td>150 ± 16</td>
</tr>
<tr>
<td></td>
<td>+ 0.1 μM VUF5455</td>
<td>2.82 ± 0.40</td>
<td>1.60 ± 0.10</td>
<td>7.25 ± 0.06</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+ 1 μM VUF5455</td>
<td>1.04 ± 0.16</td>
<td>1.48 ± 0.16*</td>
<td>6.85 ± 0.07*</td>
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</tr>
<tr>
<td><strong>CHO-A3</strong></td>
<td>Control</td>
<td>8.60 ± 0.62</td>
<td>0.78 ± 0.02</td>
<td>8.05 ± 0.04</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+ 1 μM PD81,723</td>
<td>6.78 ± 0.45*</td>
<td>1.13 ± 0.04*</td>
<td>7.78 ± 0.03*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+ 10 μM PD81,723</td>
<td>4.79 ± 0.38*</td>
<td>2.46 ± 0.18*</td>
<td>7.23 ± 0.03*</td>
<td>133 ± 4</td>
</tr>
<tr>
<td></td>
<td>+ 0.1 μM VUF5455</td>
<td>4.23 ± 0.35*</td>
<td>2.73 ± 0.22*</td>
<td>7.12 ± 0.03*</td>
<td></td>
</tr>
</tbody>
</table>

* Statistically different (p < 0.05) compared to control value, as determined by one-way ANOVA followed by a Dunnett’s multiple comparisons test.
Figure 1

(a) Diagram of the experimental setup showing reservoirs connected to a Zeiss 510 confocal microscope through an imaging cell. The setup is maintained at 37°C.

(b) Graph showing the fluorescence intensity over time for the addition and removal of a substance. The y-axis represents intensity (%), and the x-axis represents time (seconds).

(c) Graph showing the fluorescence intensity over time for a longer duration. The y-axis represents fluorescence intensity (%), and the x-axis represents time (minutes).
Figure 2

(a) Response (% 1 μM NECA) vs. [ABA-X-BY630] Log M

(b) Image of fluorescently labeled cells

(c) Fluorescence Intensity (Baseline corrected) vs. Time (minutes)

(d) Fluorescence Intensity (Baseline corrected) vs. Time (minutes)

(e) Fluorescence Intensity (%) vs. Time (minutes)

(f) Fluorescence Intensity (%) vs. Time (minutes)
Figure 5

(a) Structure diagram

(b) Simulation of fluorescence intensity over time for Infinite Dilution + VUF 5455

(c) Graph showing fluorescence intensity over time for Infinite Dilution + PD81,723

(d) Distribution of koff values for Infinite Dilution + PD81,723

(e) Graph showing fluorescence intensity over time for Infinite Dilution + VUF 5455

(f) Distribution of koff values for Infinite Dilution + VUF 5455