NanoBiT® Complementation to Monitor Agonist-Induced Adenosine A_1 Receptor Internalization

Mark Soave[1,2], Barrie Kellam[2,3], Jeanette Woolard[1,2], Stephen J. Briddon[1,2], Stephen J. Hill[1,2]

[1]Division of Physiology, Pharmacology and Neuroscience, School of Life Sciences, Queen's Medical Centre, University of Nottingham, Nottingham, NG7 2UH, UK

[2]Centre of Membrane Proteins and Receptors (COMPARE), University of Birmingham and University of Nottingham, The Midlands, UK

[3]School of Pharmacy, Centre for Biomolecular Sciences, University of Nottingham, Nottingham, NG7 2RD, UK

Correspondence to:

Stephen J. Hill, steve.hill@nottingham.ac.uk

Keywords

GPCR, Adenosine, Receptor internalization, NanoBiT, Nanoluciferase complementation.

Abstract

Receptor internalization in response to prolonged agonist treatment is an important regulator of G protein-coupled receptor (GPCR) function. The adenosine A_1 receptor (A_1AR), is one of the adenosine receptor family of GPCRs, and evidence for its agonist-induced internalization is equivocal. The recently developed NanoBiT technology utilizes split NanoLuc luciferase to monitor changes in protein interactions. We have modified the human A_1AR on the N-terminus with the small high affinity HiBiT tag. In the presence of the large NanoLuc subunit (LgBiT) complementation occurs, reconstituting a full-length functional NanoLuc luciferase. Here, we have used complemented luminescence to monitor the internalization of the adenosine A_1 receptor in living HEK293 cells. Agonist treatment resulted in a robust decrease in cell-surface luminescence, indicating an increase in A_1AR internalization. These responses were inhibited by the A_1AR-selective antagonist DPCPX with an antagonist affinity that closely matched that measured using ligand-binding with a fluorescent A_1-receptor antagonist (CA200645). The agonist potencies for inducing A_1AR internalization were very similar to the affinities previously determined by ligand-binding, suggesting little or no amplification of the internalization response. By complementing the HiBiT tag to exogenous purified LgBiT it was also possible to perform NanoBRET ligand-binding experiments using HiBiT-A_1AR. This study demonstrates the use of NanoBiT technology to monitor internalization of the adenosine A_1 receptor and offers the potential to combine these experiments with NanoBRET ligand binding assays.

Introduction

G protein-coupled receptors (GPCRs) are the largest family of membrane signalling proteins and are able to modulate signals from a wide range of endogenous ligands[1]. Prolonged stimulation by an agonist results in the internalization of many GPCRs, and this process can occur via different pathways, including caveolae-dependent and clathrin-mediated processes[2,3]. For the latter, G protein-coupled receptor kinases (GRKs) phosphorylate serine and threonine residues within the intracellular loops and C-terminal tail of the receptor following agonist-stimulated receptor activation[4]. β-Arrestins are able to bind to the phosphorylated receptor and can initiate downstream signalling pathways which are independent of G proteins[5]. β-Arrestins also compete sterically with G proteins for binding to the receptor, resulting in receptor desensitization, and recruit specific adaptor proteins which are required for clathrin-mediated endocytosis[6]. The GPCRs are internalized in clathrin-coated vesicles and transferred into early endosomes, where it is now known that a second wave of intracellular signalling can occur[7,8].

The adenosine A_1 receptor (A_1AR) is part of the wider adenosine GPCR subfamily, grouped by their ability to bind their endogenous ligand, adenosine[1,9]. The A_1AR predominantly couples to the G_i family of heterotrimeric G proteins which inhibit adenylyl cyclase mediated cAMP production. However, there is contrasting evidence concerning the nature of GRK-mediated A_1AR phosphorylation[10-13], as well as the nature and extent of A_1ARs internalization in response to chronic stimulation by agonists[12-15]. The A_1AR is able to internalize through both clathrin- and caveolae-dependent endocytosis[13,16]. Previous studies of human A_1AR internalization observed that the receptor had a slow rate of internalization over several hours[15,17-19]. Ruiz et al. (2011) found this was also true of the rat A_1AR receptor which required over 12 hours stimulation to internalize 50% of rat A_1ARs in cortical neurons[20]. These A_1AR data contrast drastically with the other G_i-coupled adenosine receptor, the A_3 receptor, which internalizes more rapidly, and within minutes of agonist stimulation[12,21,22].

Previous studies on A_1AR internalization have been conducted using either radiolabelled A_1AR ligands[14,15] or with confocal microscopy[11,19]. These techniques offer specific advantages, such as the ability to monitor A_1AR internalization in *ex vivo* tissues with radioligand binding, or the ability to directly visualize internalization with microscopy. However, these methods are intensive and low throughput.

Recently, NanoLuc binary technology (NanoBiT®) has been developed that splits the bright NanoLuc luciferase[23] into two segments, a large 18 kDa fragment (termed LgBiT), and much smaller 1.3 kDa fragment termed SmBiT (small complementation tag)[24]. These fragments have low intrinsic affinity for each other (K_D 190 μM) and complement to form the full bioluminescent protein NanoLuc. SmBiT-LgBiT complementation has successfully been used to monitor protein-protein interactions of membrane receptors, including the recruitment of G proteins and β-arrestins to GPCRs[25,26]. In the development of the NanoBiT system, other small complementary peptides were identified which have different affinities for LgBiT. One short, 11 amino acid sequence had a very high affinity for LgBiT (K_D 700 pM, termed HiBiT[24]). As an 18 kDa fragment, the LgBiT is cell impermeable and therefore HiBiT-LgBiT complementation provided an approach to distinguish between internalized proteins and those retained at the cell surface. This would not have been possible with the full-length NanoLuc luciferase. In this study, we have used A_1ARs tagged on the N-terminus with HiBiT to determine whether NanoBiT complementation can be used as a high throughput method to monitor loss of A_1ARs from the cell surface in living cells.

Materials and Methods

Materials.

Adenosine and 5'-*N*-ethylcarboxamidoadenosine (NECA) were purchased from Sigma-Aldrich (Gillingham, UK). 1,3,-Dipropyl-8-cyclopentylxanthine (DPCPX), 2-chloro-N^6-cyclopentyladenosine (CCPA), 2'methyl-2-chloro-N^6-cyclopentyladenosine (2-MeCCPA), and 2-phenylaminoadenosine (CV 1808) were obtained from Tocris (Bristol, UK). 2-Amino-6-[[2-(4-chlorophenyl)-1,3-thiazol-4-yl]methylsulfanyl]-4-[4-(2-hydroxyethoxy)phenyl]pyridine-3,5-dicarbonitrile (capadenoson) was purchased from Haoyuan Chemexpress (Shanghai, China). The fluorescent antagonist CA200645 was purchased from HelloBio (Bristol, UK). Purified LgBiT, restriction enzymes, FuGENE HD transfection reagent, and furimazine were purchased from Promega (Southampton, UK).

Constructs and Cell Lines.

To create the HiBiT-A_1AR construct, the full length NanoLuc sequence was removed from the pcDNA3.1 NLuc-A_1AR vector[27] using KpnI and BamHI restriction sites. This left the pcDNA3.1 vector containing the A_1AR with a mutated start codon (Met -> Leu). Primers containing the HiBiT sequence (bold letters), a GSSGGSSG linker (5': c**ATGGTGAGCGGCTGGCGGCTGTTCAAGAAGATTAGC**GGGAGTTCTGGCGGCTCGAG CGGTg; and 5': gatccACCGCTCGAGCCGCCAGAACTCCC**GCTAATCTTCTTGAACAGCCGCCAGCCGCT CACCAT**ggtac) and the repective KpnI and BamHI overhangs (lower case letters) were phosphorylated using T4 Polynucleotide kinase (NEB, UK) and annealed for 30 minutes at 37°C. The annealed primers were then ligated into the digested pcDNA3.1 A_1AR vector using T4 ligase (NEB, UK) creating the full-length fusion protein HiBiT-A_1AR. Correct insertion was confirmed by DNA sequencing using the School of Life Sciences Sequencing Facility at the University of Nottingham.

HEK293 cells were maintained in Dulbecco's modified eagle medium (DMEM) containing 10% fetal calf serum (FCS) and 2 mM L-glutamine at 37°C in a 5% CO_2 atmosphere. A mixed population HiBiT-A_1AR stable cell line was generated using FuGENE HD (Promega) according to the manufacturer's instructions and the cells were subjugated to three week's selection with 1 mg/mL G418.

NanoBiT Internalization Assay.

HEK293 cells stably expressing HiBiT-A_1AR were plated onto white 96-well plates (Greiner) previously coated with 10 µg/mL poly-D-lysine. 100 µL DMEM containing cells in suspension (30,000 cells/well) was added to each well and the plate incubated at 37°C in a 5% CO_2 atmosphere for 24 hours. The next day, the media was removed from each well and replaced with 50 µL HEPES-buffered saline solution (HBSS; 145 mM NaCl, 5 mM KCl, 10 mM HEPES, 1.3 mM $CaCl_2$ dihydrate, 1.5 mM $NaHCO_3$, 2 mM sodium pyruvate, 1 mM $MgSO_4.7H_2O$, 10 mM D-glucose; pH 7.45) and the relevant concentration of ligand. For end-point assays cells were incubated at 37°C for 2 hours. Purified LgBiT was diluted in HBSS (10 nM final concentration) and added to each well in the presence of furimazine (1:400 final concentration). The plate was incubated for 15 minutes in the dark at 37°C, allowing complementation to occur. Luminescence was measured on the PHERAstar FS plate reader (BMG Labtech) using the LUM Plus module.

Bioluminescence Imaging.

HEK293 cells stably expressing HiBiT-A_1AR were seeded onto a poly-D-lysine coated (10 µg/mL) 35 mm 4-chamber MatTek dish (Ashland, MA, USA) at a density of 120,000 cells/mL. The dish was incubated at 37°C in a 5% CO_2 atmosphere for 24 hours. The next day the media was removed and replaced with 400 µL HBSS containing furimazine (1:400 final concentration). Purified LgBiT was added and the plate incubated at 37°C for 20 minutes. Brightfield and bioluminescence imaging was performed on the Olympus LV200 inverted microscope (Olympus, Southend, UK). Brightfield images were captured with a 50 ms

exposure. Bioluminescence images were captured with a 45 s exposure, using a Hamamatsu EM CCD with a gain of 100.

NanoBRET Ligand-binding assay.

HEK293 HiBiT-A_1AR cells were seeded onto poly-D-lysine-coated white 96-well plates as described above. The next day, the media was removed from each well and replaced with 50 µL HBSS containing 10 nM LgBiT and the plate was incubated for 15 minutes in the dark at 37°C, allowing complementation to occur. The HBSS with unbound LgBiT was removed and replaced with 50 µL HBSS containing the fluorescent A_1-receptor antagonist ligand CA200645[28] in the absence or presence of 10 µM DPCPX. The plate was incubated in the dark at 37°C for 2 hours. Furimazine (1:400 final concentration) was added to each well and the plate incubated for 15 minutes at 37°C. The resulting BRET was measured using the PHERAstar FS plate reader (BMG Labtech) which simultaneously measured filtered light emissions at 460 (80 nm bandpass) and >610 nm (longpass). The BRET ratio was calculated by dividing the >610 nm emission by the 460 nm emission.

Data analysis.

Data were presented and analysed using Prism 7 software (GraphPad).

The potency of ligands which internalized HiBiT-A_1AR were determined from fitting data to a one-site sigmoidal concentration-response curve defined by the following four parameter logistic equation:

Eq. (1) $$\%\, receptor\ at\ cell\ surface = 100 - \frac{(100\times[A^n])}{([A^n]+IC_{50}^n)} + NS$$

where [A] is the concentration of the ligand, NS is background luminescence, *n* is the Hill coefficient, and IC_{50} is the concentration of ligand required to internalize 50% of receptors.

In the experiments where three fixed concentrations of DPCPX were used, the K_D of DPCPX was estimated from the shift in the NECA response by 10 nM DPCPX using the Gaddum equation[29]:

Eq. (2) $$\mathrm{CR} = 1 + \frac{[B]}{K_B}$$

where CR is the concentration ratio of NECA required to stimulate an identical response in the presence or absence of 10 nM DPCPX [B], and K_B is the affinity of DPCPX.

The time course of internalization in response to 10 μM NECA at HiBiT-A_1AR was fitted with a one-phase exponential decay curve using the following equation:

Eq. (3) $$Y = (Y_0 - NS)^{-k.t} + NS$$

where Y is the luminescence at time t minutes, Y_0 was the luminescence at time 0, NS is the background luminescence, and *k* is the rate constant of the decrease in luminescence per minute.

Saturation NanoBRET experiments were simultaneously fitted to obtain the total and nonspecific components using the following equation:

Eq. (4) $$BRET\ Ratio = \frac{B_{max} \times [B]}{[B] + K_D} + ((M \times [B]) + C)$$

where B_{max} is the maximal level of specific binding, [B] is the concentration of fluorescent ligand in nM, K_D is the equilibrium dissociation constant, M is the slope of the linear nonspecific binding component, and C is the y-axis intercept.

Data are presented as mean ± s.e.m. of triplicate determinations in a single experiment. In the text, *n* refers to the number of separate experiments. Statistical significance was defined as $p<0.05$ using Student's unpaired t-test.

Results

NanoLuc Binary Technology (NanoBiT) has provided a platform for assessing protein-protein interactions *in vitro* in real-time[24,30-32]. Here, we have used the NanoBiT complementation technology to monitor the presence of the human A_1-receptor, tagged on its N-terminus with HiBiT, at the cell surface of living cells following addition of purified LgBiT (Figure 1a). This approach can also be extended to detect the loss of HiBiT-A_1AR following agonist stimulation as a method to detect receptor internalization (Figure 1b).

Expression of the HiBiT-A_1AR at the plasma membrane was first confirmed using NanoBiT complementation. HEK293 cells stably transfected with HiBiT-A_1AR were incubated with purified LgBiT in the presence of the NanoLuc substrate, furimazine. As an 18 kDa protein, LgBiT is cell impermeable, and thus will only complement with HiBiT-A_1AR present on the plasma membrane. Increasing concentrations of LgBiT resulted in higher luminescence signal (Figure 2a, n=4). These results indicated that HiBiT-A_1AR was able to traffic to the plasma membrane and complement with exogenously applied purified LgBiT. It should be noted that neither HiBiT-A_1AR nor LgBiT alone produced a strong luminescent signal (Figure 2a). Widefield bioluminescent imaging also confirmed clear membrane expression of HiBiT-A_1AR in HEK293 cells (Figure 2b). From these experiments, it was determined that 10 nM LgBiT would provide a sufficient luminescence response window for all subsequent assays.

To confirm that the complementation of HiBiT-A_1AR with exogenously applied LgBiT did not alter the ability of the A_1-AR to bind ligands, ligand binding studies were performed using NanoBRET with a fluorescent A_1-antagonist[28,33] (Figure 1a). Following complementation of HiBiT-A_1AR with purified LgBiT, the binding of the fluorescent A_1-receptor antagonist CA200645 to the complemented A_1-receptor was monitored using NanoBRET[28] (Figure 1; Figure 3). In these assays, clear specific binding of CA200645 was observed to the A_1AR. The negative log of the dissociation constant (pK_D) of CA200645 was calculated to be 7.17 ± 0.03 (K_D 63.8 nM; n=4; Figure 3). This was similar to the K_D value reported previously (K_D 33.8 nM) for CA200645 to the full length NanoLuc-tagged A_1AR[27].

With successful membrane expression of HiBiT-A_1AR confirmed, it was then established if NanoBiT could be used to monitor receptor internalization (Figure 1b). Cells were incubated at 37°C and treated with 10 µM NECA for increasing periods of time to stimulate an internalization response. Longer incubation periods resulted in a decrease in luminescence (Figure 4), correlating to an increase in the proportion of HiBiT-A_1ARs which have internalized (Figure 1b). The resulting half-life of internalization was 31 ± 6 minutes (n=4). From these data, a 2-hour ligand incubation time was chosen for all subsequent end-point experiments, as receptor internalization had plateaued by this point.

NECA stimulated a concentration-dependent loss of HiBiT-A_1ARs from the cell surface of HEK293 cells (pIC_{50} of 5.67 ± 0.21, n=10; Figure 5a, Table 1). The A_1-selective antagonist DPCPX was able to inhibit the NECA-stimulated internalization response (Figure 5a). Fitting the Gaddum equation to the responses measured in the presence and absence of 10 nM DPCPX produced an affinity of DPCPX (pK_D 8.28 ± 0.12, n=6) which closely matches the affinity of DPCPX calculated at the human NLuc-A_1AR[27].

A panel of adenosine A_1 receptor agonists were screened for their ability to internalize HiBiT-A_1AR. In addition to NECA, the A_1AR agonists CCPA and 2-MeCCPA were found to be full agonists in this assay, able to stimulate a robust internalization of HiBiT-A_1AR (Figure 5b, Table 1). The atypical A_1AR agonist capadenoson, and the adenosine A_2 receptor ligand CV-1808 were found to elicit partial internalization responses (Figure 5b, Table 1).

Discussion

NanoBiT has provided the opportunity to measure GPCR-effector interactions in real-time in living cells with a high degree of sensitivity[25,26,31,32]. Here, we have used the high affinity HiBiT tag to detect cell surface expression of the adenosine A_1 receptor and quantified the loss of receptors at the cell surface in response to agonist treatment as a measure of receptor internalization.

The HiBiT-A_1AR was successfully expressed on the surface of HEK293 cells and could be visualized with bioluminescent imaging following addition of exogenous purified LgBiT. Furthermore, the complemented NLuc-A_1AR retained the ability to bind fluorescent adenosine A_1AR antagonists yielding binding constants that were similar to those determined previously using A_1ARs expressing the full length NLuc tag[27,28]. The strong luminescence signal provided by fully complemented HiBiT-LgBiT, however, provided a large assay window to detect small changes in the surface expression of the A_1AR. This can be observed in Figure 4 as the loss of luminescence in response to increasing incubation periods with the agonist NECA.

The human adenosine A_1 receptor has been shown to internalize in recombinant cell lines using radioligand binding[15,17,18]. These studies observed a slow rate of A_1AR internalization, with $t_{1/2}$ for internalization in the order of several hours. Using NanoBiT it was possible to detect A_1AR internalization (Figure 4) at much earlier time points than was previously reported[17-19]. Additionally, both radioligand binding and confocal microscopy are low-throughput techniques and not amenable to performing full concentration-response curves for a panel of A_1AR agonists.

In contrast, NanoBiT provided an ideal platform for detecting the internalization of A_1AR in living cells in response to a wide panel of ligands. The high signal-to-noise ratio of the assay made it possible to monitor full agonist and partial agonist responses over relatively short periods of time (Figure 5b). The potencies of the agonist responses were generally similar to the affinities of the ligands at the human A_1AR as measured previously with NanoBRET[27].

Thus, the similarity in values obtained for NECA and CCPA for the two assays suggests that there is no signal amplification in the internalization response for these two agonists. This contrasts with the higher receptor-effector coupling observed for these ligands for cAMP inhibition or pERK1/2 phosphorylation[34,35].

The one exception was the partial A_1AR agonist capadenoson[36,37] where the pEC_{50} (5.23) for internalization was over an order of magnitude lower that its pKi value (6.85) determined from inhibition of NanoBRET binding with CA200645 at the human A_1AR[27]. This very low potency for internalization of capadenoson compared to its binding affinity for the orthosteric ligand-binding site suggests that there may be a more complex mechanism of action involved. This may involve differential affinities for multiple A_1AR agonist receptor conformations and the potential for signalling bias[35,38,39].

The internalization stimulated by NECA could be antagonised by the A_1AR antagonist DPCPX in a concentration-dependent manner. The resulting analysis suggested an affinity for DPCPX which was in keeping with the known affinity of this ligand for the human A_1AR[27,40]. Additionally, there was no hint of inverse agonism with DPCPX in this assay (Table 1).

These experiments were performed in HEK293 cells, which express both adenosine A_{2A} and A_{2B} receptors endogenously[41]. It is unlikely that the internalization responses measured in this study were affected by the presence of the adenosine A_{2A} or A_{2B} receptors, given the potencies of the ligands used in this study are in the same rank order of the binding affinities of the human A_1AR[27,40,42], and the affinity for DPCPX determined was in keeping with the known affinity at the human A_1AR.

It should be noted that the assay described here has been configured specifically to monitor the extent of loss of A_1-receptors from the cell surface in response to agonist treatment. From the data obtained, we cannot comment on the extent to which A_1-agonists also alter A_1-receptor protein degradation and turnover. However, it should be noted that NanoBiT represents a versatile technology with a broad dynamic range which can be applied to detect

and quantify protein expression[30] and degradation[43]. For example, Riching et al. have exploited the high sensitivity of HiBiT-LgBiT complementation to detect targets with low levels of native expression, and measure their subsequent degradation following PROTAC treatment[43]. A similar approach using a HiBiT tag on the C-terminus of the A_1-receptor and its expression in cells that also express cytosolic LgBiT would allow A_1-receptor turnover and degradation to be monitored.

In conclusion, this study reports the use of NanoBiT to monitor A_1AR internalization in a plate-based assay based on complemented nanoluciferase luminescence. This approach can readily be applied to other GPCRs or indeed any cell surface membrane receptors (e.g. receptor tyrosine kinase or cytokine receptor) through the introduction of the HiBiT-tag at the extracellular terminus of the protein of interest.

Acknowledgements

This work was supported by the Medical Research Council [Grant MR/N020081/1].

Conflict of Interest

The authors declare no conflict of interest.

References

1. Alexander, S. P. H.; Christopoulos, A.; Davenport, A. P.; et al. The Concise Guide to Pharmacology 2017/18: G Protein-Coupled Receptors, *Br. J. Pharmacol* **2017**, *174*, S17-S129
2. Pierce, K. L.; Premont, R. T.; Lefkowitz, R. J., Seven-Transmembrane Receptors, *Nat. Rev. Mol. Cell. Biol.*, **2002**, *3*, 639-650
3. Calebiro, D.; Goodbole, A., Internalization of G-protein-coupled receptors: Implication in Receptor Function, Physiology and Diseases, *Best Pract. Res. Clin. Endocrinol. Metab.*, **2018**, *32*, 83-91
4. Pitcher, J.A.; Freedman, N. J.; Lefkowitz, R. J., G Protein-Coupled Receptor Kinases, *Annu. Rev. Biochem.*, **1998**, *67*, 653-692
5. Smith, J. S.; Rajagopal, S., The β-Arrestins: Multifunctional Regulators of G Protein-Coupled Receptors, *J. Biol. Chem*, **2016**, *291*, 8969-8977
6. Laporte S. A.; Oakley R.H.; Zhang J.; et al. The β2-adrenergic Receptor/β-arrestin Complex Recruits the Clathrin Adaptor AP-2 During Endocytosis, *Proc. Natl. Acad. Sci. U.S.A.*, **1999**, *96*, 3712-3717
7. Irannejad, R.; von Zastrow, M., GPCR Signaling Along the Endocytic Pathway, *Curr. Opin. Cell. Biol.*, **2014**, *27*, 109-116
8. Pavlos, N. J.; Friedman, P. A., GPCR Signaling and Trafficking: The Long and Short of It, *Trends Endocrinol. Metab.*, **2017**, *28*, 213-226
9. Fredholm, B. F.; Ijzerman, A. P.; Jacobson, K. A.; et al. International Union of Basic and Clinical Pharmacology LXXXI Nomenclature and Classification of Adenosine Receptors – an Update, *Pharm. Rev.*, **2011**, *63*, 1-34
10. Nie, Z.; Mei, Y.; Ramkumar, V., Short Term Desensitization of the A1 Adenosine Receptors in DDT 1 MF-2 Cells. *Mol. Pharmacol.*, **1997**, *52*, 456–464
11. Ferguson, G.; Watterson, K. R.; Palmer, T. M., Subtype-Specific Regulation of Receptor Internalization and Recycling by the Carboxyl-Terminal Domains of the

Human A1 and Rat A3 Adenosine receptors: Consequences for Agonist-Stimulated Translocation of Arrestin3. *Biochemistry*, **2002**, *41*, 14748–14761.

12. Klaasse, E. C.; IJzerman, A. P.; de Grip, W. J.; et al. Internalization and Desensitization of Adenosine Receptors. *Purinergic Signalling*, **2008**, *4*, 21–37.
13. Mundell, S.; Kelley, E., Adenosine Receptor Desensitizaiton and Trafficking, *Biochem. Biophys. Acta*, **2011**, *1808*, 1319-1328
14. Vendite, D.; Sanz, J. M.; López-Alañon, D. M.; et al., Desensitization of Adenosine A1 Receptor-Mediated Inhibition of Adenylyl Cyclase in Cerebellar Granule Cells. *Neurochem. Res.*, **1998**, *23*, 211–218.
15. Saura, C. A.; Mallol, J.; Canela, E. I.; et al. Adenosine Deaminase and A_1 Adenosine Receptors Internalize Together following Agonist-induced Receptor Desensitization, *J. Biol. Chem.*, **1998**, *273*, 17610-17617
16. Escriche, M.; Burgueño J.; Ciruela, F.; et al. Ligand-Induced Caveolae-Mediated Internalization of A_1 Adenosine Receptors: Morphological Evidence of Endosomal Sorting and Receptor Recycling, *Exp. Cell. Res.*, **2003**, *285*, 72-90
17. Ciruela, F.; Saura, C.; Canela, E. I.; et al. Ligand-Induced Phosphorylation, Clustering and Desensitization of A_1 Adenosine Receptors, *Mol. Pharmacol.*, **1997**, *52*, 788-797
18. Gines, S.; Ciruela, F.; Burgueño et al.; Involvement of Caveolin in Ligand-Induced Recruitment and Internalization of A_1 Adenosine Receptor and Adenosine Deaminase in an Epithelial Cell Line, *Mol. Pharmacol.*, **2001**, *59*, 1314-1323
19. Klaasse, E. C.; van den Hout, G.; Roerink, S. F.; et al., Allosteric Modulators Affect the Internalization of Human Adenosine A1 Receptors. *Eur. J. Pharmacol.*, **2005**, *522*, 1–8.
20. Ruiz, M. Á.; León, D. A.; Albasanz, J. L.; et al. Desensitization of Adenosine A1 Receptors in Rat Immature Cortical Neurons. *Eur. J. Pharmacol*., **2011**, *670*, 365–371

21. Trincavelli, M. L.; Tuscano, D.; Cecchetti, P.; et al. Agonist-Induced Internalization and Recycling of the Human A3 Adenosine Receptors : Role in Receptor Desensitization and Resensitization. *J. Neurochem.*, **2000**, *75*, 1493–1501.
22. Stoddart, L. A.; Kellam, B.; Briddon, S. J.; et al., Effect of a Toggle Switch Mutation in TM6 of the Human Adenosine A3 Receptor on G_i Protein-Dependent Signalling and G_i-Independent Receptor Internalization, *Br. J. Pharmacol.,* **2014**, *171*, 3827-3844
23. Hall, M. P.; Unch, J.; Binkowski, B. F.; et al. Engineered Luciferase Reporter From a Deep Sea Shrimp Utilizing a Novel Imidazopyrazinone Substrate. *ACS Chem. Biol.*, **2012**, *7,* 1848–57.
24. Dixon, A. S.; Schwinn, M. K.; Hall, M. P.; et al., NanoLuc Complementation Reporter Optimized for Accurate Measurement of Protein Interactions in Cells. *ACS Chem. Biol.,* **2016**, *11*: 400–408.
25. Laschet, C.; Dupuis, N.; Hanson, J., A Dynamic and Screening-Compatible Nanoluciferase-Based Complementation Assay Enables Profiling of Individual GPCR-G Protein Interactions, *J. Biol. Chem.*, **2019**, *294*, 4079-4090.
26. Storme, J.; Cannaert, A.; Van Craenenbroeck, K.; et al. Molecular Dissection of the Human A3 Adenosine Receptor Coupling with β-arrestin2, *Biochem. Pharmacol.*, **2018**, *148*, 298–307.
27. Cooper, S. L.; Soave, M.; Jörg, M.; et al. Probe Dependence of Allosteric Enhancers on the Binding Affinity of Adenosine A_1-Receptor Agonists at Rat and Human A_1-Receptors Measured Using NanoBRET, *Br. J. Pharmacol.*, **2019**, *176*, 864-878.
28. Stoddart, L. A.; Johnstone, E. K. M.; Wheal, A. J., et al.; Application of BRET to Monitor Ligand Binding to GPCRs, *Nat. Methods*, **2015**, *03*, 661–663.
29. Gaddum, J. H., The Quantitative Effects of Antagonistic Drugs., *J. Physiol.*, **1937**, *89*, 7–9

30. Schwinn, M.K.; Machleidt, T.; Zimmerman, K.; et al. CRISPR-Mediated Tagging of Endogenous Proteins with a Luminescent Peptide. *ACS Chem. Biol.* **2018**, *13*: 467–474.
31. Wan, Q.; Okashah, N.; Inoue, A.; et al., Mini G protein Probes for Active G Protein–Coupled Receptors (GPCRs) in Live Cells. *J. Biol. Chem.* **2018**, *293*: 7466–7473.
32. Yano, H.; Cai, N.S.; Javitch, J.A.; et al. Luciferase Complementation-Based Detection of G Protein-Coupled Receptor Activity. *Futur. Sci.* **2018**, *65*: 9–14.
33. Stoddart, L. A.; Vernall, A. J.; Bouzo-Lorenzo, M., et al.; Development of Novel Fluorescent Histamine H1-Receptor Antagonists to Study Ligand-Binding Kinetics in Living Cells. *Sci. Rep.*, **2018**, *8*, 1572.
34. Schulte, G.; Fredholm, B. B., Human Adenosine A_1, A_{2A}, A_{2B}, and A_3 Receptors Expressed in Chinese Hamster Ovary Cells All Mediate the Phosphorylation of Extracellular-Regulated Kinase 1/2, *Mol. Pharmacol.*, **2000**, *58*, 477-482.
35. Baltos, J. A.; Gregory, K. J.; White, P. J.; et al., Quantification of Adenosine A1 Receptor Biased Agonism: Implications for Drug Discovery, *Biochem. Pharmacol.*, **2016,** *99*, 101–112.
36. Albrecht-Küpper, B. E.; Leineweber, K.; Nell, P. G., Partial Adenosine A1 Receptor Agonists for Cardiovascular Therapies, *Purinergic Signal.*, **2012**, *8*, 91-99.
37. Tendera, M.; Gaszekewska-Zurek, E.; Parma, Z.; et al., The New Oral Adenosine A_1 Receptor Agonist Capadenoson in Male Patients with Stable Angina. *Clin. Res. Cardiol.*, **2012**, *101*, 585–591.
38. Cordeaux, Y.; Ijzerman A.P.; Hill, S.J., Coupling of the Human A_1 Adenosine Receptor to Different Heterotrimeric G-Proteins; Evidence for Agonist-Specific G-protein Activation. *Br. J. Pharmacol.* **2004**, *143***,** 705-714.
39. Draper-Joyce, C.J.; Khoshouei, M.; Thal, D.M.; et al., Structure of the Adenosine-Bound Human Adenosine A_1 Receptor-G_i Complex. *Nature*, **2018**, *558*, 559-563.

40. Bulicz, J.; Bertarelli, D. C. G.; Baumert, D.; et al. Synthesis and Pharmacology of Pyrido[2,3-*d*]pyrimidinedonies Bearing Polar Substituents as Adenosine Receptor Antagonists, *Bioorg. Med. Chem.*, **2006**, *14*, 2837-2849
41. Goulding, J.; May, L. T.; Hill, S. J., Characterisation of Endogenous A_{2A} and A_{2B} Receptor–Mediated Cyclic AMP Responses in HEK293 Cells Using the GloSensor™ Biosensor: Evidence for an Allosteric Mechanism of Action for the A_{2B}-Selective Antagonist PSB 603, *Biochem. Pharmacol*, **2018**, *147*, 55-66
42. Alnouri, M. W.; Jepards, S.; Casari, A.; et al. Selectivity is Species-Dependent: Characterization of Standard Agonists and Antagonists at Human, Rat, and Mouse Adenosine Receptors, *Purinergic Signalling*, **2015**, *11*, 389-407
43. Riching, K. M.; Mahan, S.; Corona C. R.; et al. Quantitative Live-Cell Kinetic Degradation and Mechanistic Profiling of PROTAC Mode of Action, *ACS Chem. Biol.*, **2018**, *13*, 2758-2770

Tables

Table 1 – pEC_{50} and E_{max} values of agonist-induced internalization of HiBiT-A_1AR in HEK293 cells. Data are mean ± SEM from n separate experiments. †Internalization stimulated by the highest concentration (100 μM) of this ligand ($p<0.05$ comparing E_{max} vs that of 100 μM NECA).

Ligand	**Internalization pEC_{50} (mean ± SEM)**	**E_{max} (% 100 μM NECA)**	**n**
NECA	5.67 ± 0.21	100	10
Capadenoson	5.23 ± 0.32	57.8 ± 4.4†	5
CCPA	6.43 ± 0.18	84.4 ± 6.5	10
2-MeCCPA	4.96 ± 0.13	86.7 ± 4.1	5
CV-1808	4.65 ± 0.24	47.1 ± 17.1†	5

Figure legends

Figure 1. (a) Schematic of the use of NanoBiT complementation to monitor the expression of HiBiT-A_1AR at the cell surface.A_1ARs tagged with HiBiT on their N terminus are expressed at the cell surface of HEK293 cells. Upon addition of exogenous purified LgBiT, complementation occurs between HiBiT and LgBiT, forming the full-length NLuc luciferase. In the presence of the substrate, furimazine, the complemented NLuc is luminescent. Addition of a red A_1-receptor fluorescent ligand such as CA200645 that binds to the HiBiT-tagged A_1AR allows NanoBRET to occur between the NLuc and the fluorescent ligand. (b) Schematic of the NanoBiT internalization assay. Under basal conditions, N-terminally HiBiT-tagged A_1ARs are expressed on the plasma membrane of HEK293 cells. Upon addition of exogenous purified LgBiT, complementation occurs between HiBiT and LgBiT, forming the full-length NLuc luciferase. In the presence of the substrate, furimazine, luminescence is read as the experimental output. When treated with an agonist, the HiBiT-A_1AR undergoes internalization and is removed from the plasma membrane. After two hours, purified LgBiT is added to the media, but it cannot cross the plasma membrane and complement with the HiBiT-A_1AR, and therefore there is less complemented NLuc as a result.

Figure 2. (a) The effect of purified LgBiT titrations on complemented luminescence in HEK293 cells stably expressing HiBiT-A_1AR following 15 minutes incubation with furimazine (1:400). Bars indicate the luminescence measured by 200 nM LgBiT (open bar) and HiBiT-A_1AR (closed bar) alone, respectively. Data are mean ± SEM from triplicate determinations in a single experiment. This single experiment is representative of four independent experiments performed. (b) Widefield bioluminescence imaging of HEK293 cells stably expressing HiBiT-A_1AR with increasing concentrations of purified LgBiT. Cells were incubated with LgBiT and furimazine (1:400) for 20 minutes prior to imaging. Images are representative of three separate experiments. Scale bars show 20 µm.

Figure 3. NanoBRET ligand binding at the A_1AR using fully complemented HiBiT-LgBiT. 10 nM LgBiT was first added to HEK293 cells stably expressing HiBiT-A_1AR before addition

of increasing concentrations of CA200645 for two hours. Non-specific binding was determined in the presence of 10 μM DPCPX. Data are mean ± SEM from triplicate determinations in a single experiment. This single experiment is representative of four independent experiments performed.

Figure 4. Timecourse of HiBiT-A_1AR internalization in HEK293 cells. Luminescence of cell surface complemented HiBiT-LgBiT in cells treated with 10 μM NECA for up to 4 hours. Data are mean ± SEM of triplicate determinations from a single experiment. This single experiment is representative of five separate experiments.

Figure 5. (a) Ability of DPCPX to inhibit the NECA-induced internalization response in HiBiT-A_1AR. Internalization of HiBiT-A_1AR in response to NECA in the absence or presence of the antagonist DPCPX at 10, 30, 100 nM, respectively. Data are mean ± SEM from triplicate determinations in a single experiment. This single experiment is representative of six separate experiments. (b) Concentration response curves of HiBiT-A_1AR internalization stimulated by a panel of A_1-receptor agonists in HEK293 cells. Internalization is reported as a percentage of internalization stimulated by a two hour incubation with 100 μM NECA. Data are mean ± SEM from least five separate experiments, where each experiment was performed in triplicate. The number of individual experiments performed with each agonist is given in Table 1.

Figure 1

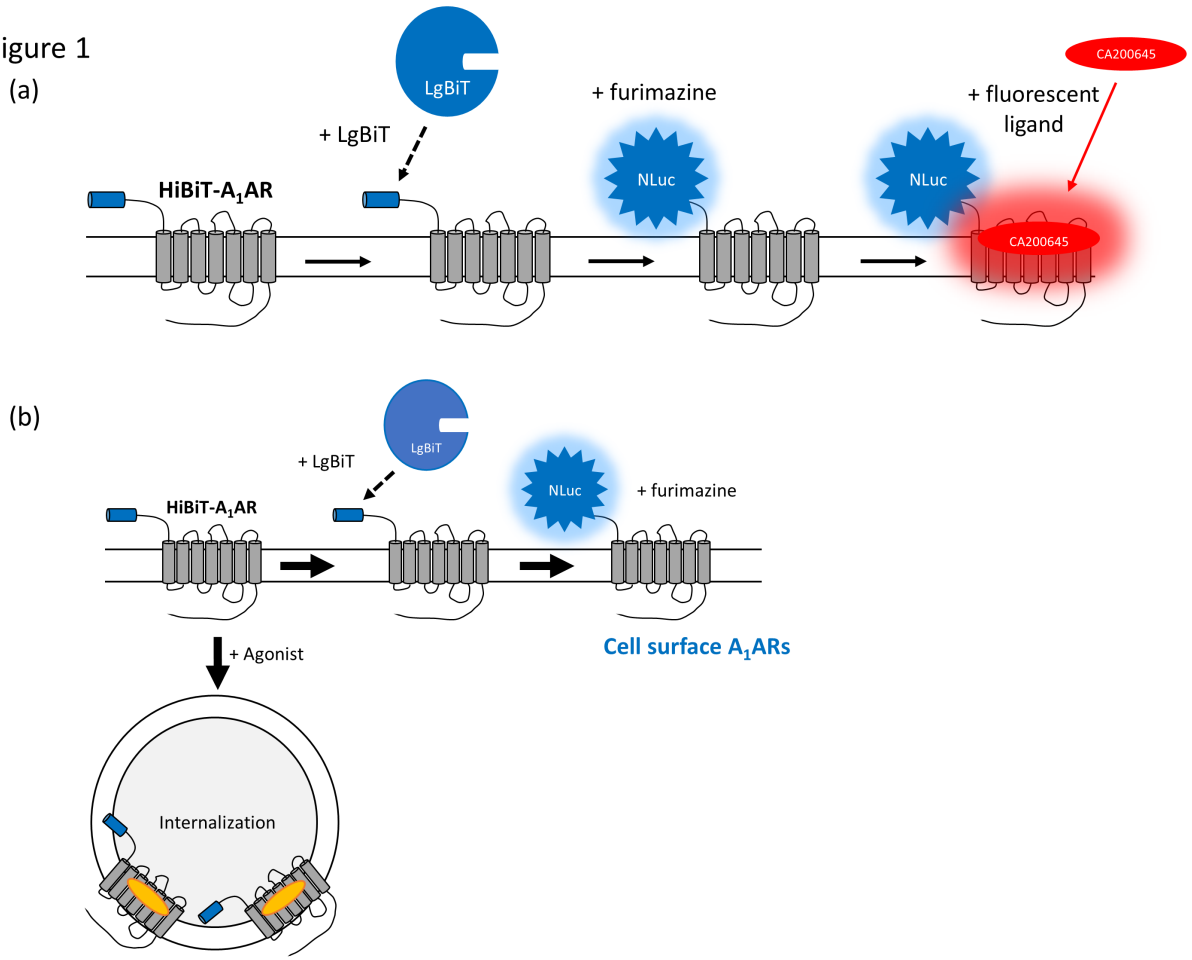

(a)
LgBiT
+ LgBiT
HiBiT-A1AR
+ furimazine
NLuc
NLuc
CA200645
+ fluorescent ligand
CA200645
(b)
LgBiT
+ LgBiT
HiBiT-A1AR
NLuc
+ furimazine
Cell surface A1ARs
+ Agonist
Internalization

Figure 2.

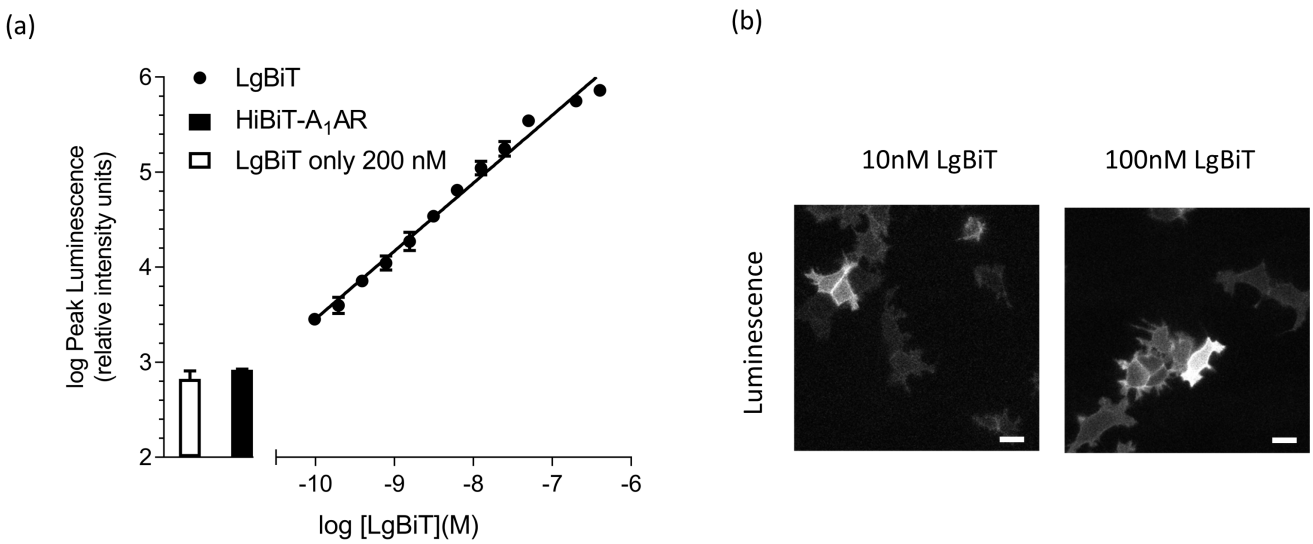

(a)
LgBiT
HiBiT-A1AR
LgBiT only 200 nM
log Peak Luminescence (relative intensity units)
6
5
4
3
2
-10
-9
-8
-7
-6
log [LgBiT](M)
(b)
10nM LgBiT
100nM LgBiT
Luminescence

Figure 3.

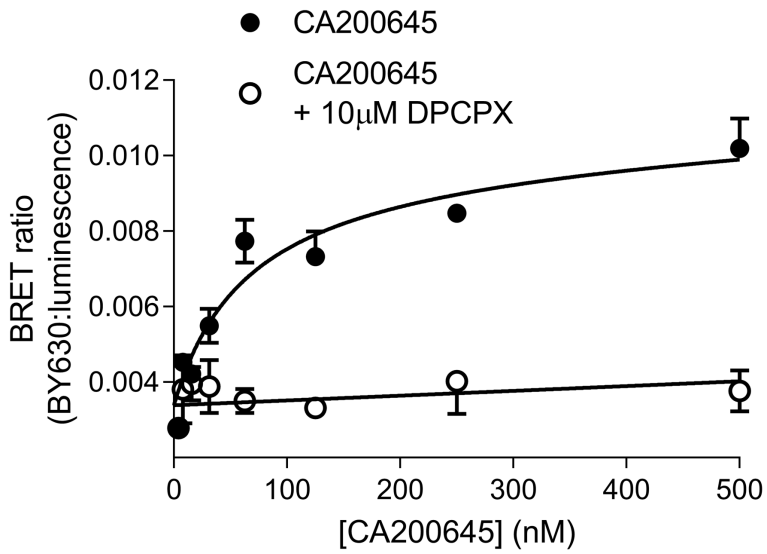

CA200645
CA200645
+ 10μM DPCPX
BRET ratio
(BY630:luminescence)
0.012
0.010
0.008
0.006
0.004
0
100
200
300
400
500
[CA200645] (nM)

Figure 4.

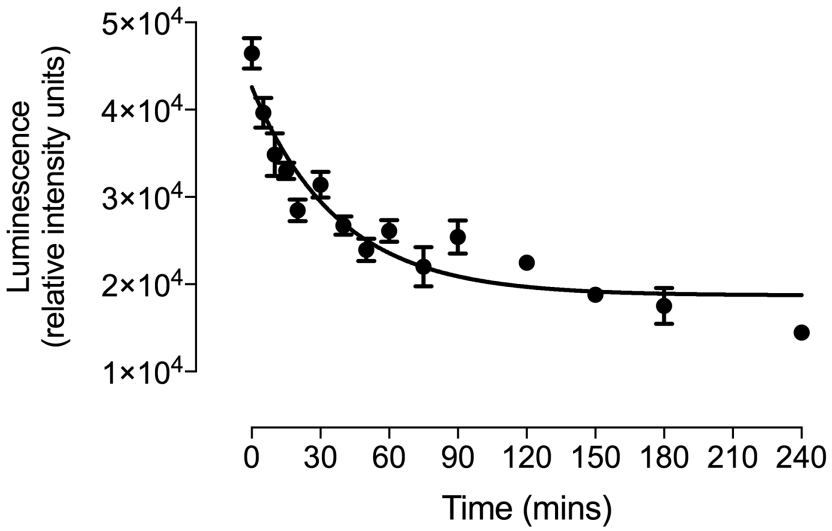
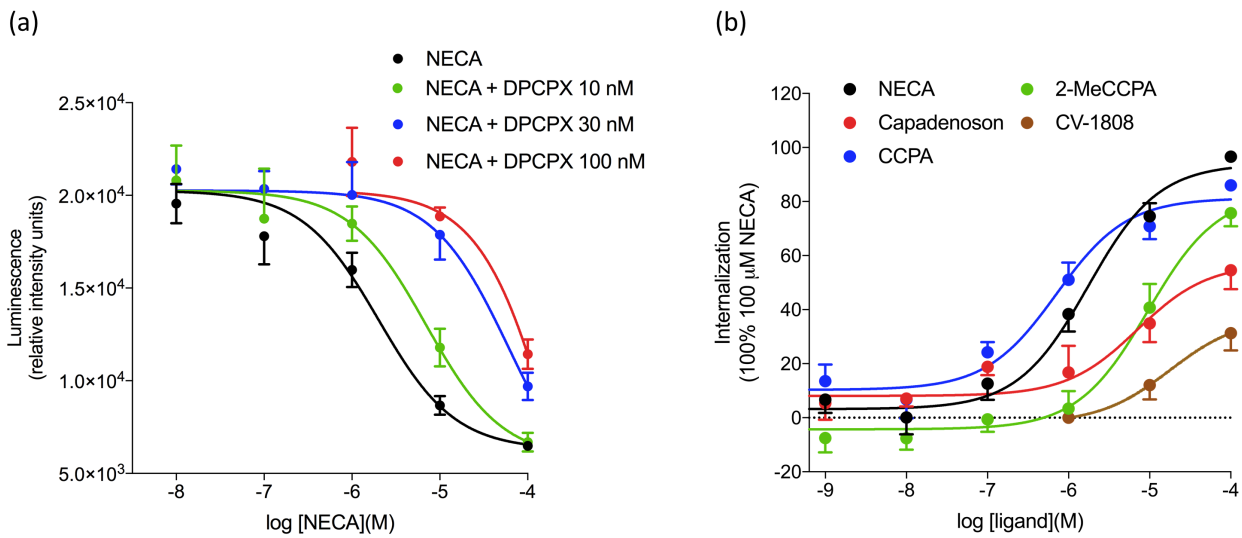

Luminescence
(relative intensity units)
5×10^4
4×10^4
3×10^4
2×10^4
1×10^4
0
30
60
90
120
150
180
210
240
Time (mins)

Figure 5.


(a)
NECA
NECA + DPCPX 10 nM
NECA + DPCPX 30 nM
NECA + DPCPX 100 nM
Luminescence (relative intensity units)
2.5×10^4
2.0×10^4
1.5×10^4
1.0×10^4
5.0×10^3
-8
-7
-6
-5
-4
log [NECA](M)
(b)
NECA
Capadenoson
CCPA
2-MeCCPA
CV-1808
Internalization (100% 100 μM NECA)
120
100
80
60
40
20
0
-20
-9
-8
-7
-6
-5
-4
log [ligand](M)