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Application of BRET to monitor ligand binding to the adenosine A_1 and A_3 receptors in living cells

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Energy transfer techniques have long been used to monitor protein-protein interactions but have not been widely used to investigate drug-protein interactions. Bioluminescence resonance energy transfer (BRET) using a luminescent protein Nanoluc (Nluc) (1) from the deep sea shrimp, *Olophorus*, has recently been used to develop purified bioluminescent sensor proteins to detect drug concentrations in human serum (2). This technique used a tethered ligand but with the development of fluorescent ligands for G protein-coupled receptors (GPCRs) (3) means they could potentially be used directly as BRET acceptors. Here, we describe the development of a BRET assay that can measure ligand binding to the adenosine A_1 and A_3 receptors in living cells.

Full length cDNA encoding the A_1 or A_3 receptor was amplified and fused in frame into a vector containing Nluc to generate Nluc-A_1 and Nluc-A_3. These constructs were transfected into HEK293 cells and stable cell lines generated. Cells were grown to confluence on white walled, clear bottomed 96-well plates. Cells were then incubated with the required concentration of fluorescent ligand and competing ligand for 1 h at 37°C. The Nluc substrate, furmazine (10 µM) was then added to each well and the luminescence and resulting BRET measured using the PHERAstar FS plate reader (BMG Labtech). Filtered light emissions were measured at 460 nm (80 nm bandpass) and at 535 nm (60 nm bandpass) for BYFL labelled ligands and at 460 nm (80 nm bandpass) and >610 nm (longpass) for BY630 labelled ligands. All data are mean ± SEM of four separate experiments performed in triplicate.

Initially, saturation binding experiments on cells expressing Nluc-A_1 and Nluc-A_3 were carried out with increasing concentrations of the BODIPY 630/650 labelled antagonist, CA200645 (4). For both receptors a clear saturable signal with low levels of non-specific binding was observed and the equilibrium dissociation constant (K_D) was calculated as 7.48 ± 2.4 nM for Nluc-A_1 receptor and 7.6 ± 3.7 nM for Nluc-A_3. To confirm the specificity of the signal, the A_3-selective antagonist AV039 (BY630 linked) (5) was used in saturation experiments with both receptors. No specific signal was observed at Nluc-A_1 but a specific signal was observed at Nluc-A_3 (K_D at Nluc-A_3 = 24.6 ± 8.3 nM). A specific signal was also observed with a BODIPY-FL linked fluorescent ligand, XAC-ser-tyr-BYFL (6), at both Nluc-A_1 and Nluc-A_3 (K_D = 167.0 ± 74.4 nM for Nluc-A_1 and 7.6 ± 3.7 for Nluc-A_3). CA200645, AV039 and XAC-ser-tyr-BYFL were also used in competition binding experiments with a range of unlabelled adenosine receptor antagonists. The calculated affinity values (K_i) of each of the compounds using all three labelled antagonists was in the expected range. For example, the A_3 selective antagonist MRS1220 displaying higher affinity at Nluc-A_3 (pK_i = 9.2 ± 0.1) than Nluc-A_1 (pK_i = 7.1 ± 0.2) and vice versa for the A_1 selective antagonist DPCPX (pK_i = 8.4 ± 0.1 at Nluc-A_1 and 6.9 ± 0.1 at Nluc-A_3).

Here we have demonstrated that fluorescent ligand-Nluc BRET can be used for both saturation and competition ligand binding assays in living cells without the

requirement for wash steps. This assay has the potential to be widely used to study many aspects of ligand binding at GPCRs.

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