Adenosine A_{2A} Receptor Ligand Binding Experiments by Using Real-time Single-cell FRET

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[Abstract] We designed a fluorescence resonance energy transfer (FRET)-based approach to study the ligand binding constants of the adenosine A_{2A} receptor ($A_{2A}R$). Our assay is based in the interaction of a fluorescent $A_{2A}R$ agonist ligand (MRS5424) with an $A_{2A}R$ tagged with the cyan fluorescent protein (CFP) at the N-terminus (*i.e.* $A_{2A}R^{CFP}$) and expressed in living cells. Thus, upon fast superfusion of the $A_{2A}R^{CFP}$ expressing cells with MRS5424, the ligand-receptor interaction is determined by single-cell FRET in a real-time mode. Accordingly, our approach allowed immediate 'real-time' readout of the ligand-receptor interaction, thus allowing kinetic binding experiments, a feature impossible to achieve using conventional radioisotope-labelled ligands. In addition, since our assay permitted the visual confirmation of receptor localization it also allowed localized saturation binding experiments.

Materials and Reagents

1. Cell line (*i.e.* HEK-293 cells)
2. Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich)
3. Sodium pyruvate
4. L-glutamine
5. Antibiotics: streptomycin and penicillin
6. Fetal bovine serum
7. TransFectin™ Lipid Reagent (Bio-Rad Laboratories)
8. Hank's balanced salt solution (HBSS) (see Recipes)
9. Cell culture medium (see Recipes)

Equipment

1. 18 mm diameter glass coverslips
2. Attofluor holder
3. Inverted Axio Observer microscope (ZEISS) equipped with a 63x oil immersion objective

4. Polychrome V (TILL Photonics)
5. Avalanche photodiodes (TILL Photonics)
6. Focal drug application system (ALA Scientific Instruments, OCTAFLOW^{TM})
7. Digidata 1440A analog/digital converter (Molecular Devices)

Software

1. pCLAMP (Molecular Devices)
2. GraphPad Prism (GraphPad Software)

Procedure

1. Two days before the experiment, the cells (*i.e.* HEK-293 cells) were seeded onto 18 mm diameter glass coverslips and transiently transfected with an $A_{2A}R$ construct tagged with the CFP at its N-terminal tail ($A_{2A}R^{CFP}$) (Figure 1).

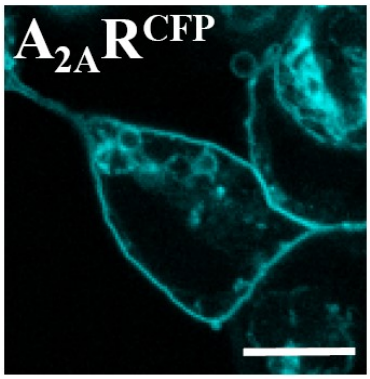

Figure 1. Cell surface localisation of the $A_{2A}R^{CFP}$ construct. HEK-293 cells were transiently transfected with $A_{2A}R^{CFP}$, fixed and analyzed by confocal microscopy. The $A_{2A}R^{CFP}$ was mainly targeted to the cell surface and scarcely accumulated at the intracellular level (Fernández-Dueñas *et al.*, 2013). Scale bar: 10 μm

2. The day of the experiment the transiently transfected cells were mounted in an Attofluor holder and placed on an inverted Axio Observer microscope equipped with a 63x oil immersion objective and a dual-emission photometry system.
3. Then, cells were continuously superfused with a FRET-compatible $A_{2A}R$ fluorescent ligand (*i.e.* MRS5424) (Fernández-Dueñas *et al.*, 2012) dissolved in HBSS and applied with the aid of a focal drug application system.
4. A Polychrome V was used as the light source in our dual-emission photometry system. Upon excitation with the corresponding donor excitation wavelength (*i.e.* $A_{2A}R^{CFP}$) the

fluorescent signals of the donor and acceptor fluorophores were detected by avalanche photodiodes and digitized using a Digidata 1440A analog/digital converter.

5. pCLAMP and GraphPad Prism softwares were used for data collection and analysis.
6. Accordingly, a FRET signal was measured upon donor (*i.e.* $A_{2A}R^{CFP}$) excitation at 430 ± 10 nm [beam splitter dichroic long-pass (DCLP) 460 nm] and an illumination time set to 10 ms at 10 Hz. Then, the emission light intensities were determined at 535 ± 15 nm (F_{535}; MRS5424 emission) and 480 ± 20 nm (F_{480}; $A_{2A}R^{CFP}$ emission) with a beam splitter DCLP of 505 nm. No corrections for spillover between channels or direct MRS5424 excitation were made.
7. The increase in FRET ratio (F_{535}/F_{480}) was fitted to the equation: $r(t) = A \times (1 - e^{-t/\tau})$, where τ is the time constant (in seconds) and A is the magnitude of the FRET signal (Figure 2). When necessary for calculating τ, agonist-independent changes in FRET due to photobleaching were subtracted (Fernández-Dueñas *et al.*, 2012, Fernández-Dueñas *et al.*, 2013).

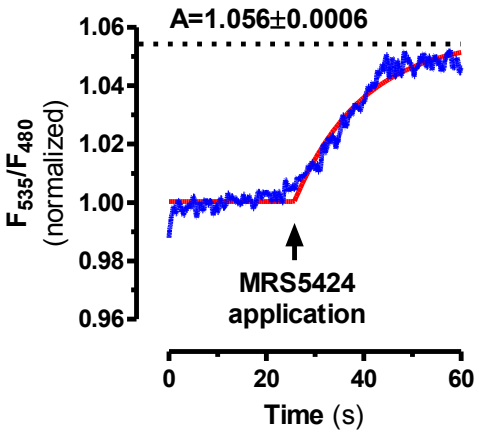

Figure 2. Example of FRET ratio fitting. Time-resolved changes in $A_{2A}R^{CFP}$ and MRS5424 fluorescence emission signals in single cells transfected with $A_{2A}R^{CFP}$ (see Figure 1). The ratio (blue trace) of the emission intensities of the MRS5424 (F_{535}) and CFP (F_{480}) in response to MRS5424 application was recorded from single HEK293 cells expressing the $A_{2A}R^{CFP}$ (see Figure 1). Shown are the changes induced by rapid superfusion with 2 M MRS5424. The increase of the ratio F_{535}/F_{480} was fitted by a simple monoexponential curve ($r(t) = A \times (1 - e^{-t/\tau})$) using the GraphPad Prism software which gave a time constant (τ) in this experiment of 14 ± 1 s. This assay is well suited for competitive ligand binding experiments using non-fluorescent compounds (Fernández-Dueñas *et al.*, 2012, Fernández-Dueñas *et al.*, 2013).

Recipes

1. HBSS
   137 mM NaCl
   5.4 mM KCl
   0.3 mM Na_2HPO_4
   0.4 mM KH_2PO_4
   4.2 mM $NaHCO_3$
   1.3 mM $CaCl_2$
   0.5 mM $MgCl_2$
   0.6 mM $MgSO_4$
   5.6 mM glucose
   pH 7.4
2. Cell culture medium
   Dulbecco's modified Eagle's medium (DMEM) supplemented with:
   1 mM Sodium pyruvate
   2 mM L-glutamine
   100 U/ml streptomycin
   100 mg/ml penicillin
   5% (v/v) fetal bovine serum

Acknowledgments

This work was supported by grants SAF2011-24779, Consolider-Ingenio CSD2008-00005 and PCIN-2013-019-C03-03 from Ministerio de Economía y Competitividad and ICREA Academia-2010 from the Catalan Institution for Research and Advanced Studies (to FC), by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) Intramural Research Program (to KAJ). FC belong to the "Neuropharmacology and Pain" accredited research group (Generalitat de Catalunya, 2014 SGR 1251). We thank E. Castaño and B. Torrejón from the Scientific and Technical Services (SCT) group at the Bellvitge Campus of the University of Barcelona for their technical assistance.

References

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