European Journal of Pharmacology 788 (2016) 122–131

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Molecular and cellular pharmacology

Stabilizing effects of G protein on the active conformation of adenosine A_1 receptor differ depending on G protein type

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ARTICLE INFO

Article history:
Received 26 February 2016
Received in revised form
15 June 2016
Accepted 15 June 2016
Available online 16 June 2016

Keywords:
G protein coupled receptor
Adenosine A_1 receptor
Förster resonance energy transfer (FRET)
Conformational change
G protein coupling

ABSTRACT

G protein coupled receptors (GPCRs) trigger various cellular and physiological responses upon the ligand binding. The ligand binding induces conformational change in GPCRs which allows G protein to interact with the receptor. The interaction of G protein also affects the active conformation of GPCRs. In this study, we have investigated the effects of $G_{\alpha i1}$, $G_{\alpha o}$ and chimeric $G_{\alpha qi5}$ on the active conformation of the adenosine A_1 receptor, as each G_α showed difference in the interaction with adenosine A_1 receptor. The conformational changes in the adenosine A_1 receptor were detected as the agonist-induced decreases in efficiency of Förster resonance energy transfer (FRET) between fluorescent proteins (FPs) fused at the two intracellular domains of the adenosine A_1 receptor. Amplitudes of the agonist-induced FRET decreases were subtle when the FP-tagged adenosine A_1 receptor was expressed alone, whereas they were significantly enhanced when co-expressed with $G_{\alpha i1}G_{\beta 1}G_{\gamma 2}2$ (Gi1) or $G_{\alpha qi5}G_{\beta 1}G_{\gamma 2}2$ (Gqi5) but not with $G_{\alpha o}G_{\beta 1}G_{\gamma 2}2$ (Go). The enhancement of the agonist-induced FRET decrease in the presence of Gqi5 was significantly larger than that of Gi1. Furthermore, the FRET recovery upon the agonist removal in the presence of Gqi5 was significantly slower than that of Gi1. From these results it was revealed that the agonist-bound active conformation of adenosine A_1 receptor is unstable without the binding of G protein and that the stabilizing effects of G protein differ depending on the types of G protein.

1. Introduction

G protein coupled receptors (GPCRs) are important signaling molecules that trigger the downstream signaling and cause various cellular responses through activating heterotrimeric G protein upon the binding of their ligands, such as neurotransmitters, hormones and lipids. The ligand binding induces conformational changes of GPCRs, resulting in the interaction with and stimulation of heterotrimeric G protein. Recently, crystallographic studies have revealed three dimensional structures of the ligand bound form of various GPCRs (Hulme, 2013). Especially in β-adrenoceptor, the inactive structure and the activated structures with or without G protein have been solved (Cherezov et al., 2007; Rasmussen et al., 2011a, 2011b; Rosenbaum et al., 2007, 2011). Comparison between those structures shed light on the activation mechanisms of GPCR by G protein. The agonist binding makes the intracellular binding pocket to be accessible for G protein (Rasmussen et al., 2011b) and the G protein binding is considered to fully open the binding pocket (Nygaard et al., 2013).

The ligand-induced conformational changes in GPCRs have been analyzed by the optical method (Lohse et al., 2012). Activation of the receptors was shown to decrease the efficiency of Förster resonance energy transfer (FRET) between the fluorescent proteins (FPs) fused at the intracellular third loop (i3) and the C-tail of family A GPCRs, such as α_{2A}-adrenoceptor (Vilardaga et al., 2003), the muscarinic receptors (Markovic et al., 2012; Tateyama and Kubo, 2013b) and the metabotropic purinergic $P2Y_1$ receptor (Tateyama and Kubo, 2013a). As one FP is attached to the flexible C-tail, the decreases in FRET are assumed as the increases in distance between the i3 and C-tail, consistent with the structural studies. Stabilizing effects of G protein on the active conformation of receptors are also demonstrated by the FRET analyses using the functionally intact FP-tagged receptors. The inhibition of the Gi/o coupling, by the pertussis toxin (PTX), attenuated the agonist-induced FRET decrease in the Gi/o coupled α_{2A}-adrenoceptor (Vilardaga et al., 2003), and co-expression of Gq protein increased the amplitudes of the FRET decreases in the Gq coupled muscarinic receptors (Tateyama and Kubo, 2013a, 2013b). In addition to the amplitudes of the FRET decreases, the concentration-response curves were also affected by the G protein bindings,

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http://dx.doi.org/10.1016/j.ejphar.2016.06.025
0014-2999/

consistent with studies of the ligand binding assay (Flynn et al., 1989).

The active conformation of GPCRs is stabilized by formation of the ternary complex (the agonist, the active receptor and G protein, A/R*/G) (Christopoulos and Kenakin, 2002), which raised the possibility that the affinity of G protein to the activated receptor is a key determinant for the stabilizing effects. Here we examined this possibility by monitoring the agonist-induced changes in the intra-subunit FRET efficiency of the adenosine A_1 receptor in the absence or the presence of the different types of G protein, such as $G_{\alpha i1}$, $G_{\alpha o}$ and the chimeric $G_{\alpha qi5}$, whose affinities to the adenosine A_1 receptor are expected to be different (Berstein et al., 1992; Jockers et al., 1994).

2. Materials and methods

2.1. Molecular biology and expression system

cDNAs for the adenosine A_1 receptor, the dopamine D_2 receptor, $G_{\alpha i1}$ and $G_{\alpha o}$ were isolated from mouse brain cDNA library by PCR (Tateyama and Kubo, 2013b). The chimeric $G_{\alpha qi5}$ was constructed by exchanging the last five residues of $G_{\alpha q}$ with those of $G_{\alpha i1}$ (Conklin et al., 1993), as the GTP incorporation of $G_{\alpha qi5}$ was expected to be slower than that of $G_{\alpha i1}$ (Berstein et al., 1992). YFP or CFP was attached to the C-tail of the tested receptors (receptor-FP). For the FRET analysis of the intra-subunit conformational changes, YFP with junctional linkers was fused at Asn^{221}/Pro^{222} of A_1R-CFP. Used as the linkers were three glycin residues (-*GGG*-YFP-*GGG*-, A_1R-YC-3G) or parts of i3 residues of the α_{2A}-adrenoceptor (-*PDACSAPPGGADRRGPNGL*-YFP-*SGHGEERGGGAKAS*-, A_1R-YC-long). CFP was attached to the N-tail of $G_{\beta 1}$ (Tateyama and Kubo, 2013b). YFP with junctional linkers (SGGGS) was fused at Ile^{93}/Asp^{94} of $G_{\alpha i1}$ and at Phe^{123}/Glu^{124} of $G_{\alpha qi5}$. The cDNA construct of YFP fused with PH domain (YFP-PH) was kindly gifted from Dr. Jalink (van der Wal et al., 2001). HEK293T cells were seeded on the glass treated with poly-L-Lysine and cultured with D-MEM supplemented with 10% fetal bovine serum, antibiotics and 4 mM L-glutamine. HEK293T cells were transfected with the constructs subcloned into the pcDNA3.1(–) expression vector using Lipofectamine2000 (Invitrogen), as previously described (Tateyama and Kubo, 2013b). 24–48 h after the transfection, the fluorescent images, fluorescent signals or whole cell currents were recorded from 1 to 5 cells in each combination of the constructs. Independent experiments were carried out more than two times for each combination of the constructs and then the data were summarized. In some experiments, PTX (300 ng/mL) was added to the culture medium for 14–24 h.

2.2. TIRF image acquisition and FRET analysis

The fluorescence images were acquired every three seconds using a total internal reflection fluorescence (TIRF) microscope and a cooled CCD camera (Micromax, Roper Scientific), as previously described (Tateyama and Kubo, 2013b). Intensity of the FRET efficiency was evaluated as a ratio of net FRET (nF) to the intensity of CFP (nF/I_{CFP}). Cells on the glass bottom dish were perfused with the bath solution (140 mM NaCl, 1 mM $CaCl_2$, 4 mM KCl, 0.3 mM $MgCl_2$, 10 mM HEPES, pH 7.4 adjusted with NaOH). Dopamine (DA) and oxotremorine M (oxo-M) were used as the agonists of dopamine D_2 receptor and muscarinic M_2 receptor, respectively. Various concentration of an agonist of the adenosine A_1 receptor, 5′-N-ethylcarboxamidoadenosine (NECA), was applied through exchanging the perfused solution.

2.3. Electrophysiology

The functional Gi/o coupling of the tested constructs was evaluated by recording the whole-cell current through the G protein gated inwardly rectifying potassium (GIRK) channels (Tateyama and Kubo, 2011) from cells co-transfected with the GIRK1 and GIRK2 constructs. The internal solution was composed of 140 mM KCl, 4 mM Na_2-ATP, 3 mM EGTA, 10 HEPES, 5 $MgCl_2$ (pH 7.3 adjusted with KOH). GTP (0.3 mM) was also supplemented before the patch clamp experiments. Cells expressing YFP were held at −80 mV, and then concentrations of KCl and NaCl in the bath solution were changed 140 mM and 4 mM, respectively. Exchange of the various concentrations of NECA was performed by using fast perfusion system (Tateyama and Kubo, 2013b).

2.4. Fluorometry

For the high time resolution FRET analyses, the photometric recording were performed, as described previously (Tateyama and Kubo, 2013b). Briefly intensities of CFP and FRET (I_{CFP} and I_{FRET}) were simultaneously detected by two photomultiplier tubes and acquired by the Digidata and pClamp 9 software (Axon Instruments) at 5 kHz. Similarly, the incorporation of the BODIPY® TR-GTP (Texas Red, Invitrogen) into the YFP fused G_α subunits (G_α-YFP) was monitored as FRET between YFP and TR (Murakoshi et al., 2004). Instead of GTP, TR-GTP (5 μM) was solved in the internal solution and applied into the cells expressing G_α-YFP through the glass pipette. During the fluorometric recording, cells were held at 0 mV to eliminate the possible influences of the membrane potential on the conformation of the adenosine A_1 receptor. Five minutes after the rapture of membrane, YFP was excited by light pass through an excitation filter (488–512 nm). The emitted light was divided by a 564 dichroic mirror and then intensities of YFP (I_{YFP}, emission filter 510–550 nm) and TR ($I_{TR\text{-}FRET}$, emission filter 590–630 nm) were simultaneously recorded as described above. To evaluate efficiencies of FRET, I_{FRET} and $I_{TR\text{-}FRET}$ were normalized by I_{CFP} and I_{YFP}, respectively. The time-lapse FRET changes induced by the application and removal of NECA were fitted to a single exponential function to elucidate the time constants.

2.5. Statistics

The ligand-induced changes in $I_{pH\text{-}YFP}$ and nF/I_{CFP} were evaluated as previously described (Tateyama and Kubo, 2013b). The EC_{50} values were estimated by fitting the concentration response curve to a Hill equation. All data are expressed as means ± S.E.M., with n indicating the number of data. A statistical significance between two groups was estimated by unpaired Student's *t*-test and that between more than two groups was by Tukey's test; values of $P < 0.05$ were considered as statistically significant.

3. Results

3.1. Different Gα subunits bind to the adenosine A_1 receptor with different affinities

In this study, we aimed at investigating the effects of different Gα subunits whose affinities to the receptors are different. For this purpose, we first analyzed the binding of G protein to the receptors by monitoring FRET between $G_{\beta 1}$ tagged with CFP (CFP-$G_{\beta 1}$) and the receptors fused with YFP. The fluorescent intensities of YFP were measured under the TIRF illumination and were not different between A_1R-YFP, D_2R-YFP and M_2R-YFP (Table 1), indicating that the YFP-fused receptors express on the surface