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FLOW CYTOMETRY APPLICATION FOR STUDIES ON ADENOSINE A_{2A} RECEPTORS EXPRESSION

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Abstract: Adenosine A_{2A} receptors belong to the heptaspanning membrane receptors family A, also known as G protein-coupled receptors. In human brain they are highly expressed in striatum, where they co-exist and co-function with adenosine A_1, glutamate $mGlu_5$ and dopamine D_2 receptors. As glutaminergic neurotransmission modulators in GABAergic enkephalinergic neurons, adenosine A_{2A} receptors are attractive targets for new, alternative therapies of neurodegenerative disorders, like Parkinson's disease and Huntington's disease. The aim of the research was to obtained fluorescently tagged adenosine A_{2A} receptors. Gene encoding human adenosine A_{2A} receptor was inserted into plasmid pEYFP-N1, bearing enhanced yellow fluorescent protein (EYFP). The construct was expressed in HEK 293 cells. Fluorescence was observed by flow cytometry and epifluorescence microscopy. Functional ligand binding properties were investigated by saturation binding analysis of adenosine A_{2A} receptors specific agonist [^{3}H] CGS 21680.

Keywords: flow cytometry, adenosine A_{2A} receptors, fusion proteins, radioligand binding assays, fluorescence microscopy

Adenosine plays important role not only intracellulary, but also as extracellular signal compound. In the central neural system, adenosine modulates sleep, locomotion, nociception, drug addiction and other vitally important processes. Adenosine mediates its actions through specific G protein-coupled receptors, which are classified into four subtypes: A_1, A_{2A}, A_{2B} and A_3 receptors (1). A_1 and A_{2A} receptors bind adenosine with high affinity (k_d 0.1 – 1.0 μM), whereas A_{2B} and A_3 receptors have lower affinity for adenosine (k_d 10 μM) (2). Adenosine A_{2A} receptors are localized in the central nervous system, specially in striatum, where they modulate glutamatergic neurotransmission via the GABAergic enkephalinergic neurons (3). At the presynaptic side, they are localized in active zone, forming heteromeric complexes mostly with adenosine A_1, but also with glutamatergic $mGlu_5$ and dopaminergic D_2 receptors. At the postsynaptic side they are localized at the perisynaptic ring adjacent to the postsynaptic density, forming heteromeric complexes with glutamatergic $mGlu_5$ and dopaminergic D_2 receptors. As G_s, G_{olf} proteins-coupled receptors, adenosine A_{2A} receptors transmit signal intracellulary, through adenyl cyclase, protein kinase A cascade (4). A_{2A} and D_2 receptors regulate the function of the GABAergic enkephalinergic neurons by antagonistic interactions (5, 6). Consequently, by blocking adenosine A_{2A} receptors, dopaminergic transmission can be increased. This effect can be used in the new, alternative therapies of some neurodegenerative disorders, specially Parkinson's disease (7). To date, there is no drug with adenosine A_{2A} receptor antagonistic properties in Parkinson's disease available at the European market, but some compounds are being evaluated in clinical trials and one (Istradefylline) may reach the US market this year (8).

Green Fluorescent Protein (GFP) (9) and its variants have become one of the most widely used tools to study cellular distribution and trafficking of proteins of interest. Genetic engineering of fluorescent proteins leads to different spectral properties, increased brightness and folding efficiences, as compared with the wild types (10). Attachment of such proteins to the G protein-coupled receptors has become widely applied method to study receptors in living cells (11). Moreover, interactions between receptors and other elements can be investigated in

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detail. For example, interaction with ligands with agonistic/antagonistic properties (12), as well as with G proteins can be visualized, as well as interaction with β-arrestines (13). Another area of interests, which has been widely investigated in recent years is cross-talk between receptors of the same type (homodimerization) or different types (heterodimerization) (14). The best established ways to measure this type of protein-protein interactions are resonance energy transfer and protein complementation assays. In both approaches both partners require the attachment of the reporter proteins. In resonance energy transfer assays one reporter is donor fluorophore or bioluminescent protein and the other reporter is longer wavelength acceptor fluorophore. The efficiency of non-radiative transfer between donor and acceptor protein is determinated by the distance between them (15). In protein complementation assays the reporters are halves of a protein, which can only function when the two fragments are brought together and fold around each other.

In the area of instrumentation, a great variety of tools is presently offered. Steady-state fluorescence spectroscopy can be applied to get fluorescence emission spectra from cells population in

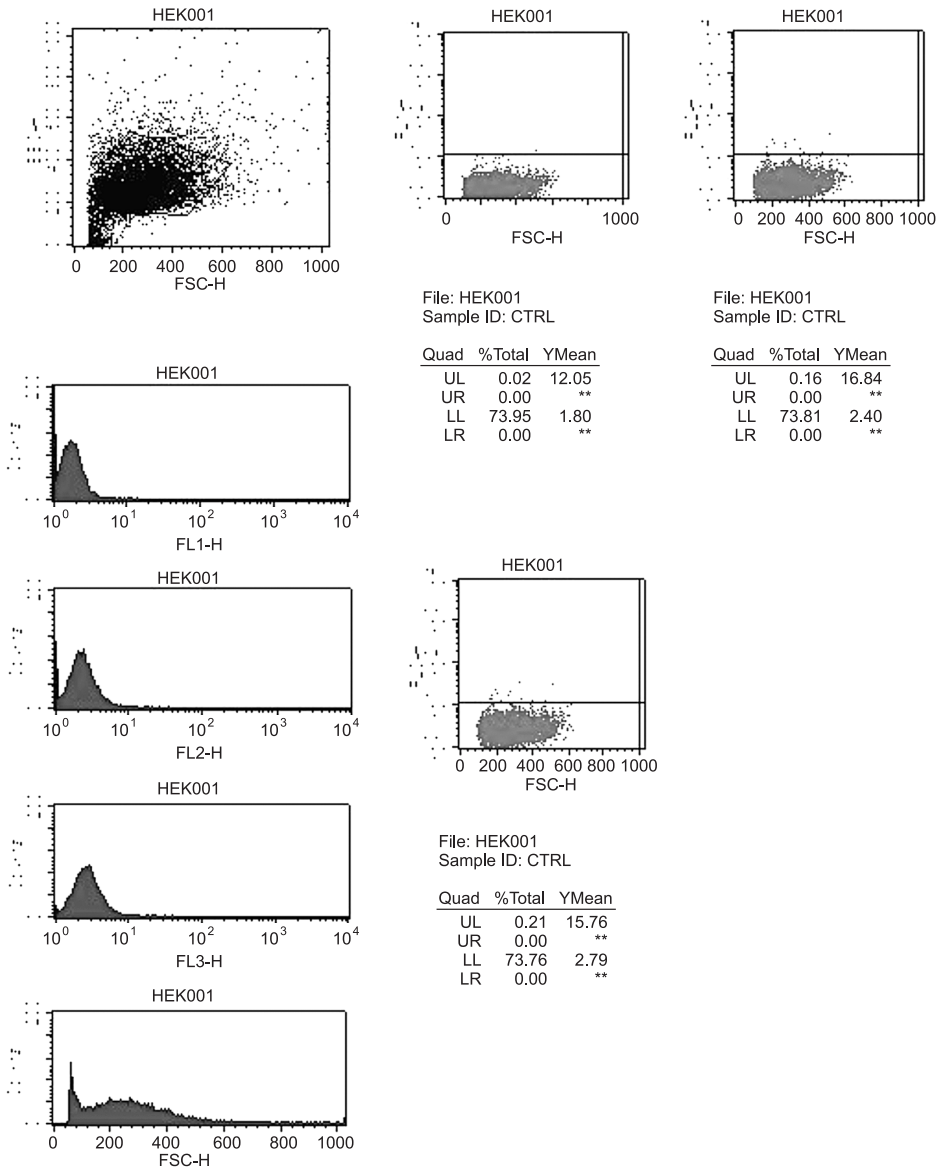

Figure 1. Flow cytometric profile of non-transfected HEK293 cells.

solution. Flow cytometry offers fast and exact measurements of each cell. Such parameters like number, shape, viability, mobility, together with fluorescent properties can be measured. Fluorescent resonance energy transfer can be also measured by flow cytometry (16). In order to visualize fluorescent proteins fusion constructs distribution and trafficking in living cells fluorescent microscopy has to be applied. Confocal microscopy offers better resolution, comparing with white-field epifluorescence microscopy and a possibility to measure energy transfer by acceptor photobleaching. Fluorescence lifetime imaging microscopy can provide quantitative information, since fluorescence lifetime measurements are independent of any change of fluorophore concentration or excitation intensity.

EXPRIMENTAL

Plasmids

The pcDNA 3.1 plasmid, encoding human adenosine A_{2A} receptor (UMR cDNA Resource Center, Rolla, MO) was PCR-amplified using an universal primer for pcDNA 3.1 and a reverse primer without stop codon and carrying EcoRI

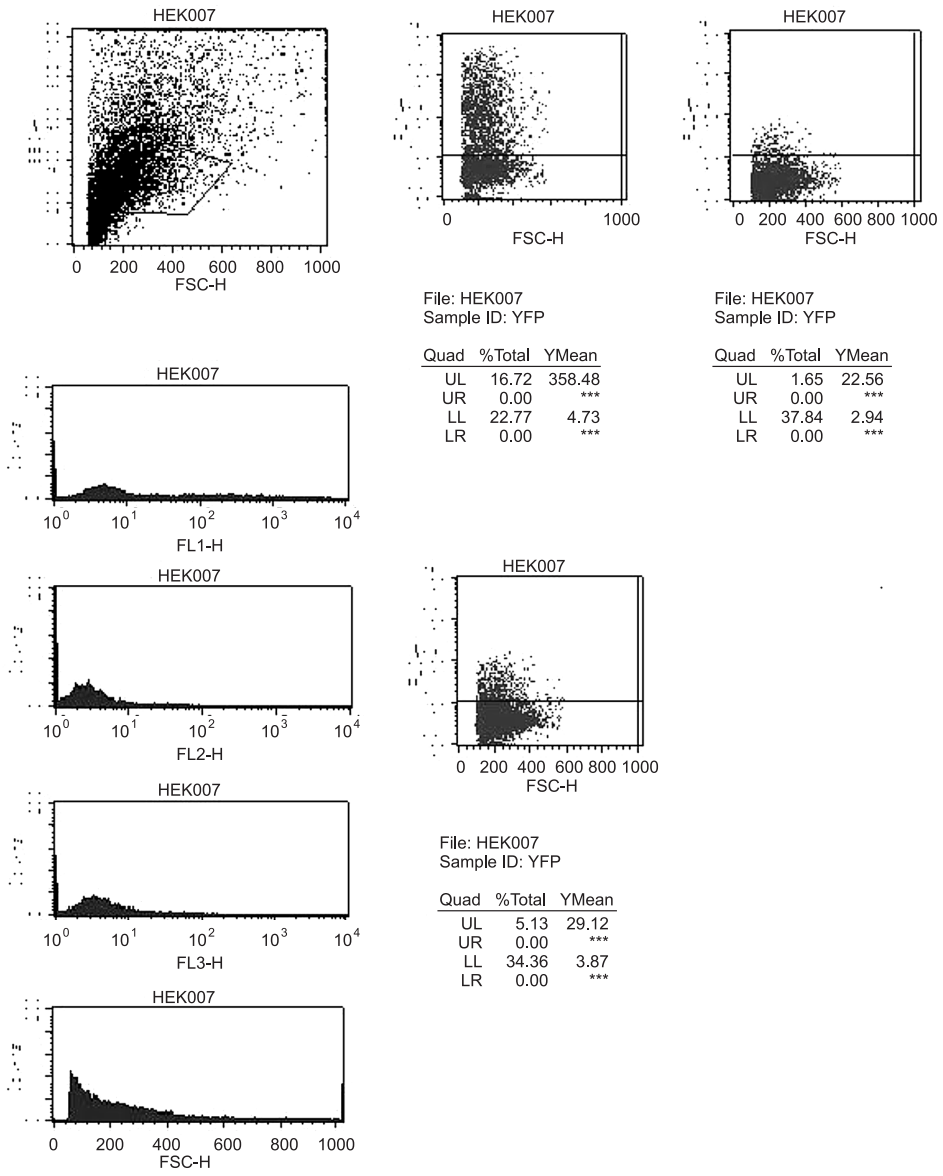

Figure 2. Flow cytometric profile of HEK293 cells transfected with EYFP-N1 plasmid.

restriction site. The entire coding sequence was inserted into pEYFP-N1 plasmid (Clontech, Palo Alto, CA).

Cell cultures and transfection

Human embronic kidney 293 (HEK293) cells were cultured at 37°C and 5% CO_2 in DMEM medium (Sigma), supplemented with 10% heat-inactivated FBS (Sigma) and 1% L-glutamine (Sigma). Cells were transiently transfected using FuGene HD reagent (Roche) for radioligand binding assays or using Lipofectamine (Invitrogen) for epifluorescence microscopy, accoring to the manufacturers instructions. 48 h after transfection cells were harvested for following experiments.

Epifluorescence microscopy

Cells were grown on poly(D-lysine)-treated coverslips (number 0) and transiently transfected with 2 µg of plasmid. Coverslips were placed into a microscope chamber containing physiological HEPES-buffered saline solution (130 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM HEPES, and 10 mMD-glucose, pH 7.4). Cells were then imaged using an inverted Nikon TE2000-E microscope (Nikon Instruments, Melville, NY)

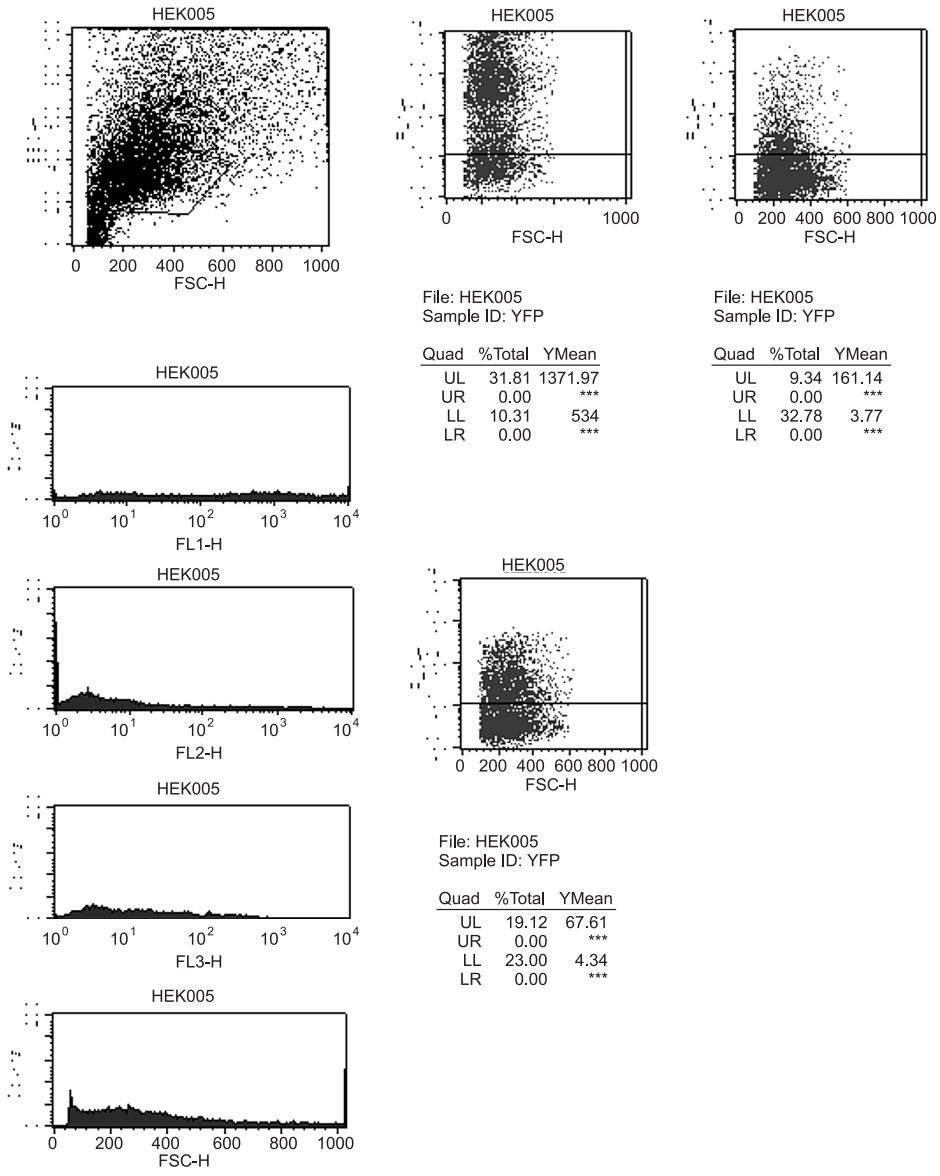

Figure 3. Flow cytometric profile of HEK293 cells transfected with adenosine A_{2A} receptor EYFP fusion construct.

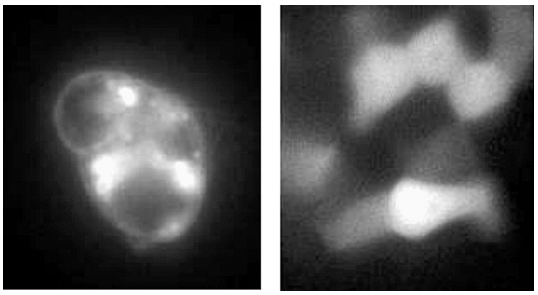

Figure 4. Fluorescence microscopy images of HEK293 cells transfected with adenosine A_{2A} receptor-EYFP fusion construct (left) and EYFP plasmid alone (right).

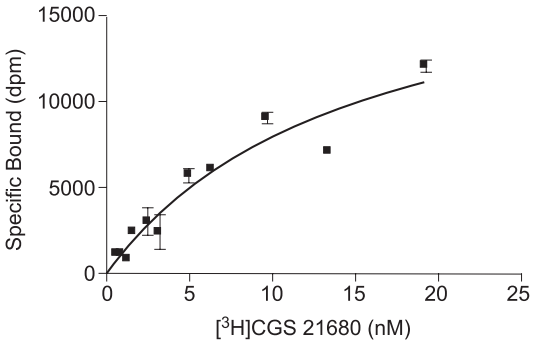

Figure 5. Saturation binding [^{3}H] CGS 21680 to human adenosine A_{2A} receptors fused with EYFP fluorescent proteins, expressed in HEK293 cells.

equipped with a 40 (numerical aperture 1.3) oilimmersion Fluor lens and a cooled digital photometrics CoolSnap-HQ charge-coupled device camera (Roper Scientific, Trenton, NJ). Epifluorescence excitation light was generated by an ultra-highpoint intensity 75 W xenon arc Optosource lamp (Cairn Research, Faversham, Kent, UK) coupled to a computer-controlled Optoscan monochromator (Cairn Research). Monochromator was set at 495/9 nm.

Radioligand binding assay

Radioligand binding assay was performed as described in (17). Briefly, the transfected HEK 293 cells were washed with phosphonate-buffered saline (PBS), scraped from dish in PBS and centrifuged at 1000 rpm for 5 min. Pellets were resuspended in 50 mM Tris-HCl, pH 7.4, containing 10 mM $MgCl_2$, using an Ultra Turrax homogenizer. This homogenate was recentrifuged at 48000 × g for 10 min at 4^{o}C. The resulting pellet was resuspended in buffer containing 2 IU/mL of adenosine deaminase (Type III, Boehringer Mannheim, Indianapolis, IN) to 20 mg/mL of original tissue weight and incubated at 37^{o}C, in order to remove endogenous adenosine. This membrane homogenate was recentrifuged and the final pellet was used for the assay. The saturation studies were carried out in triplicate, in tubes containing 100-200 µg of protein per mL in incubation buffer. Tubes were incubated with 10 different concentrations of [^{3}H]CGS 21680 ranging from 0.5 to 50 nM. Nonspecific binding was defined in the presence of 20 µM 2-CADO. Binding reactions were terminated by filtration through Whatman GF/B filters. Filters were washed twice with ice-cold buffer and placed in scintillation vials and bound radioactivity was determined by liquid scintillation counting (Beckman LS 650). Estimation of the radioligand binding parameters was carried out using the GraphPad Prism 2.0 program (GraphPad Software, San Diego CA).

Flow cytometry

Cells were transiently transfected using a calcium phosphonate precipitation procedure (18). 48 h after transfection cells were harvested by trypsynization and resuspended in phosphonate buffer saline with 1% bovine serum albumin. Data were collected with FACSCalibur flow cytometer (BD Biosciences, San Jose, CA), equipped with the argon ion laser (wavelength of 488 nm) and a 635 nm Spectrum laser. Signals were detected with 530/30 nm bandpass filter (into FL1), 585/42 nm bandpass filter (into FL2) and 670LP nm bandpass filter (into FL3).

RESULTS

The aim of this study was to develop adenosine A_{2A} receptor fusion protein, which can be used in various fluorescent-based assays. pEYFP-N1 plasmid, bearing enhanced yellow fluorescent protein variant was employed and adenosine A_{2A} receptor was cloned into it. Obtained construct was subcloned in *Escherichia coli* DH5a bacteria. In order to get receptors expression in their functional form, human HEK 293 cells were transiently transfected with obtained construct by different methods, like calcium phosphonate precipitation, FuGene HD or Lipofectamine reagent. HEK 293 are cells of choice in these type of experiments, because they offer high fidelity of exogenous receptors expression, rapid cell division and low patch-clamping (19). Flow cytometric profile of non-transfected HEK 293 cells (Figure 1) shows homogeneous population of non-fluorescent cells. For cells transfected with pEYFP plasmid (Figure 2), fluorescence is observed in all detection channels, but the highest one is observed in FL1 channel, what agrees with fluorescence emission maximum for yellow fluorescent protein. For cells transfected with pEYFP plasmid, with adeno-

sine A_{2A} receptor encoding gene (Figure 3), the highest fluorescence is observed, comparing with cells transfected with pEYFP plasmid without the insert. Epifluorescence imaging reveals membrane localization of adenosine A_{2A} receptors-EYFP fusion proteins (Figure 4, left), comparing with diffuse expression of enhanced yellow fluorescent proteins alone (Figure 4, right), although, significant fluorescence of fusion construct is observed in the cells organelles.

In order to investigate the effect of the presence of fluorescent tag on receptors function, saturation binding of specific adenosine A_{2A} receptors agonist [^{3}H]CGS 21680 was performed (Figure 5). The C-terminal tag did not influence the binding parameters. The obtained k_d values (14.9 nM) are close to the values shown for these receptors in the rat brain (17).

DISCUSSION

The C-terminally tagged adenosine A_{2A} receptors were expressed in HEK 293 cells, as judged both by flow cytometry and by epifluorescence microscopy imaging techniques. Most of the expressed constructs were localized in the plasma membrane, although significant fraction of expressed constructs were localized in endoplasmic reticulum and Golgi apparatus. This may indicate misfolding of some of the receptors constructs or that portion of the receptor proteins is still on the way to the final destination in the cell membrane. Saturation binding experiments show that the fraction of receptors construct present in the plasma membrane is fully functional and able to bind specific ligands. Continuing efforts to investigate obtained constructs expression, advanced microscopic techniques like confocal microscopy and fluorescence lifetime imaging are planned to be used.

The EYFP, like most of fluorescent proteins available at the market have natural tendency to self associate (20). This can be blocked with the A206K point mutation in the amino acids sequence. The monomeric fluorescent proteins, like for example mCitrine (21) can replace the EYFP as fusion partners and it may improve folding of fusion proteins constructs.

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