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Search for adenosine A_{2A} spare receptors on peripheral human lymphocytes

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ABSTRACT

Some ligand–receptor couples involve spare receptors, which are apparent when a maximal response is achieved with only a small fraction of the receptor population occupied. This situation favours cross-reactions with low-affinity ligands, which may be detrimental for cell signaling. In the case of the adenosine A_{2A} receptors ($A_{2A}R$), which have an immunosuppressive effect on lymphocytes through cAMP production, the presence of spare $A_{2A}R$ remains to be established. We examined the situation using patients over-expressing lymphocyte $A_{2A}R$ and an agonist-like mAb to $A_{2A}R$. We found that maximal mAb binding and functional response varied among the patients whereas the dissociation constant and half-maximal effective concentration had similar mean values (0.19 and 0.18 μM, respectively). Lymphocyte $A_{2A}R$ expression was correlated to plasma adenosine level and $A_{2A}R$ occupation but not to $A_{2A}R$ response. These results are consistent with a lack of a reserve of functional $A_{2A}R$ on human lymphocytes as a general rule and suggest that the amount and functional state of the expressed $A_{2A}R$ determine the maximal level of the lymphocyte response to adenosine.

1. Introduction

Adenosine, an endogenous purine nucleoside, acts through four classes of G protein-coupled receptors (A_1R, $A_{2A}R$, $A_{2B}R$ and A_4R) to exert various physiologic effects [1]. Most of these actions affect cardiovascular, neuronal and immune cells [2–7]. In lymphocytes, adenosine regulates multiple physiologic processes including inflammation [8–14] and congenital defect of adenosine deaminase – an enzyme that degrades the nucleoside and hence regulates its plasma level – associated with aberrant signaling through adenosine receptors contributes to the severe combined immunodeficiency syndrome [15]. $CD4^+$ and $CD8^+$ T-cells express $A_{2A}R$, $A_{2B}R$, and A_3R [16–21]. Numerous in vivo and in vitro studies suggest that $A_{2A}R$ selectively inhibit T-cell receptors of activated T-cells, thereby inhibiting lymphocyte inflammatory activity [18,22–25]. Activation of the $A_{2A}R$ on $CD4^+$ T-cells prevents myocardial ischemia–reperfusion injury by inhibiting the lymphocyte accumulation and activation in the reperfused heart [26]. $A_{2A}R$ can also prevent Th1/Th2 development [27] as well as T-cell apoptosis [28]. Finally, it was reported that adenosine produced by regulatory T-cells mediates immune suppression activity through $A_{2A}R$ [29,30]. Thus, the study of the expression and function of $A_{2A}R$ on lymphocytes appears to be of pivotal importance to evaluate their immuno-regulatory role.

Various ligand–receptor models are available in pharmacology and may be applicable to agonist–$A_{2A}R$ interactions in order to study the role of $A_{2A}R$ in lymphocyte regulation. Among them, the spare receptor theory [31] postulates that a given ligand can exert maximum biological effect while occupying only a small amount of the available receptors. Such a reserve of receptors may allow for a response to low, transient ligand concentrations and low-affinity interactions which are compatible with several T-cells properties, hence supporting the presence of spare receptors for T-cell receptor activation [32]. From a pharmacological point of view, the presence of spare receptors is evidenced if the maximal ligand response (E_{max}) is obtained at less than the maximal occupation of the receptors (B_{max}). This pattern is usually determined by comparing the half-maximal effective concentration (EC_{50}) that refers to the ligand concentration necessary to reach 50% of the maximal effect with the dissociation constant (K_D) that refers to the ligand concentration necessary to reach 50% of the maximal binding. If the EC_{50} is less than the K_D, spare receptors are existing.

Spare $A_{2A}R$ were reported in cardiac tissue of guinea pig using an irreversible $A_{2A}R$ antagonist to block receptor response to various agonists [33]. In other study on mouse T-cells, using gene targeting to generate mice lacking one or two alleles of $A_{2A}R$, the authors showed that the decrease in the number of $A_{2A}R$ in thymocytes from $A_{2A}R^{+/-}$ and $A_{2A}R^{-/-}$ mice versus the $A_{2A}R^{+/+}$ control mice resulted

Abbreviations: $A_{2A}R$, adenosine A_{2A} receptors; E_{max}, maximal ligand response; B_{max}, maximal occupation of the receptors; EC_{50}, half-maximal effective concentration; K_D, dissociation constant; NMS, neurally-mediated syncope; APC, adenosine plasma concentration; PBMC, peripheral blood mononuclear cells; AU, arbitrary units

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in proportional decrease in the adenosine-induced cAMP and apoptosis responses of their respective T-cells [34]. No investigations were conducted on human lymphocyte $A_{2A}R$ expression, occupation and function because of some experimental constraints, mainly low $A_{2A}R$ expression in normal individuals and lack of efficient ligand to stably bind to the receptor. Here, we took advantage of Adonis, an agonist-like mAb which steadily binds to an extra-cellular part of the $A_{2A}R$ [35], to investigate the presence of a reserve of $A_{2A}R$ in peripheral lymphocytes of patients with neurally-mediated syncope (NMS) who generally display high adenosine plasma concentrations (APC) and increased lymphocyte $A_{2A}R$ expressions [36–38].

2. Materials and methods

2.1. Selection of patients

Ten patients with clinical symptoms of NMS [38] were enrolled. NMS patients were chosen because they frequently exhibit high APC [36,39] associated with high expression levels of $A_{2A}R$ on peripheral lymphocytes [37], which was found to mirror the increase in $A_{2A}R$ expression on disease target organs [40]. This study was conducted in compliance with the principles of the Declaration of Helsinki and approved by the Ethics Committee for Human Research of our university hospital. All patients provided written informed consent to participate.

2.2. Adenosine assay

Blood sampling was processed as described using laboratory-prepared tubes containing 3 ml of cold-stop solution composed of inhibitors of degradation and red blood cell uptake of adenosine [36,39,41]. Samples were then maintained on ice until centrifugation. After deproteinization, adenosine was quantified by HPLC (Chrom-Systems, Munich, Germany), adenosine being identified by its elution time and spectrum [39]. Measurement was made by comparison of peak areas with those obtained using standards. The intra-assay and inter-assay coefficients of variation ranged from 1% to 3%.

2.3. Incubation of Adonis with lymphocytes

Blood samples were collected from brachial vein into 8 ml tubes containing sodium citrate/Ficoll (BD Vacutainer CPT, Beckton Dickinson, Franklin lakes, NJ). Peripheral blood mononuclear cells (PBMC) were prepared according to the manufacturer's instructions. Viable cell recovery was consistently over 98% with more than 95% of lymphocytes and less than 3% granulocytes. 0.25×10^6 cells were mixed with Adonis (0–1.2 μM in 1 ml culture medium), an IgM mAb directed against a linear part of the second external loop of $A_{2A}R$ [35]. After 90 min incubation at room temperature under shaking, PBMC were either washed twice with phosphate-buffered saline, pH 7.3 for binding and $A_{2A}R$ expression tests or only centrifuged without washing for functional test. Cell pellets were kept at −20 °C until use.

2.4. Western blotting

To quantify both Adonis binding to lymphocytes and the lymphocyte $A_{2A}R$ expression, we used the Western blotting procedure previously described [42]. PBMC pellets previously incubated with or without Adonis were lysed by a 3 min sonication treatment at 47 kHz with the SDS–PAGE loading buffer containing 5% mercaptoethanol. Samples were then submitted to 12% SDS–PAGE analysis followed by transfer onto a PVDF membrane. Filters were incubated 20 min with Adonis and then with horseradish peroxidase-labeled anti-mouse IgG Fab specific antibodies prior to chemiluminescent staining with SuperSignal West Femto (Pierce Biotechnology, Rockford, IL).

In SDS–PAGE analysis in reducing condition, Adonis resolved in heavy (65 kDa) and light (25 kDa) IgM chains bands. Quantification of Adonis bound to $A_{2A}R$ was based on the detection of the light chain of the IgM because it is readily detected by the labeled second antibody directed partly to the light chain of Fab portion of mouse IgG shared by mouse IgM. Densitometry analysis used ImageJ 1.42q (National Institutes of Health, USA) and results from duplicates were expressed as arbitrary units (AU) defined as pixels of the light chain band versus blot background.

The lymphocyte $A_{2A}R$ expression was similarly quantified except that AU were defined as pixels of the canonical $A_{2A}R$ band versus blot background. $A_{2A}R$ from PBMC not previously incubated with Adonis migrated at 45 kDa and the corresponding band was detected by Adonis binding during the Western blot procedure.

Adonis binding curves were established using six concentrations of Adonis and non linear regression analysis were performed with a one site specific binding equation $Y = B_{max} \times X/(K_D + X)$ using Prism 5 (GraphPad Software, Inc La Jolla, CA). B_{max} expressed using the same units as Y values and K_D expressed using the same units as X values were estimated for Adonis binding to each PBMC preparation.

2.5. cAMP test

The agonist properties of Adonis allow its use as a specific effector for cAMP production [35]. Briefly, cellular cAMP measurement was achieved on PBMC pellets incubated with Adonis by ELISA using the Amersham Biotrak Kit (GE healthcare Bio-Sciences, Upsala, Sweden). Stimulation was stopped by adding dodecyltrimethylammonium bromide acetate buffer. Results of duplicates were expressed as AU defined as cAMP production generated in the presence versus in the absence of Adonis. We examined the results obtained using six concentrations of Adonis with Prism 5 (GraphPad Software) and the sigmoidal dose–response curve: $Y = \text{Bottom} + (\text{Top} - \text{Bottom})/(1 + 10^{[(\text{Log EC}_{50} - X) \times \text{Hill Slope}]})$. Top ($E_{max}$ here) and Bottom are plateau values in the units of Y and EC_{50} is the concentration of Adonis that gives a response half way between Top and Bottom values. Hill slope describes the steepness of the family of curves.

2.6. Statistical analysis

Quantitative variables were expressed using means ± SEM. Paired t-test was used to compare K_D and EC_{50} values and correlation tests were made between adenosine, B_{max} and E_{max} values. All the tests were two-tailed and P-values less than 0.05 were considered as statistically significant. Analyses were performed with Prism 5 (GraphPad Software).

3. Results

3.1. Adenosine levels

The patients listed in Table 1 (7 women and 3 men; mean age: 52 years) were selected on the basis of NMS clinical symptoms. They had APC ranging from 0.29 to 2.91 μM with a mean value ± SEM of 1.29 ± 0.27 μM. As expected from the literature on NMS, most patients (7 out of 10) had higher APC than control subjects (0.20 to 0.70 μM; data from the Biochemistry Laboratory of the Timone Hospital, Marseille, France).

3.2. Saturation curves and dose–response curves

Adonis binding to PBMC from patients was assessed by Western blotting visualizing the light chain of bound Adonis (Fig. 1(A)). After densitometry analysis of the bands, the resulting Adonis binding curves exhibited various plateau values ranging from 3.05 to 68.13 AU (pixels of the light chain band versus blot background) with a mean