Search for adenosine A2A 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 A2A receptors (A2A R), which have an immunosuppressive effect on lymphocytes through cAMP production, the presence of spare A2A R remains to be established. We examined the situation using patients over-expressing lymphocyte A2A R and an agonist-like mAb to A2A 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 A2A R expression was correlated to plasma adenosine level and A2A R occupation but not to A2A R response. These results are consistent with a lack of a reserve of functional A2A R on human lymphocytes as a general rule and suggest that the amount and functional state of the expressed A2A R determine the maximal level of the lymphocyte response to adenosine.

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1. Introduction

Adenosine, an endogenous purine nucleoside, acts through four classes of G protein-coupled receptors (A1R, A2AR, A2BR and A3R) 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 A2AR, A3AR, and A1R [16–21]. Numerous in vivo and in vitro studies suggest that A2AR selectively inhibit T-cell receptors of activated T-cells, thereby inhibiting lymphocyte inflammatory activity [18,22–25]. Activation of the A2AR on CD4+ T-cells prevents myocardial ischemia–reperfusion injury by inhibiting the lymphocyte accumulation and activation in the reperfused heart [26]. A2AR 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 A2AR [29,30]. Thus, the study of the expression and function of A2AR 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–A2AR interactions in order to study the role of A2AR 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 (EC50) 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 EC50 is less than the K_d, spare receptors are existing.

Spare A2AR were reported in cardiac tissue of guinea pig using an irreversible A2AR 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 A2AR, the authors showed that the decrease in the number of A2AR in thymocytes from A2AR+/− and A2AR−/− mice versus the A2AR+/+ control mice resulted

Abbreviations: A2AR, adenosine A2A receptor; E_max, maximal ligand response; B_max, maximal occupation of the receptors; EC50, 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 A₂AR expression, occupation and function because of some experimental constraints, mainly low A₂AR 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 stably binds to an extra-cellular part of the A₂AR [35], to investigate the presence of a reserve of A₂AR in peripheral lymphocytes of patients with neurally-mediated syncope (NMS) who generally display high adenosine plasma concentrations (APC) and increased lymphocyte A₂AR 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 A₂AR on peripheral lymphocytes [37], which was found to mirror the increase in A₂AR 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. Viability of cell recovery was consistently over 98% with more than 95% of lymphocytes prepared according to the manufacturer’s instructions. Viable PBMC (10⁶ cells/well) were added to 24-well plates containing 1 ml culture medium, an IgM mAb directed partly to the light chain of Fab portion of mouse IgG shared by mouse IgM. Densitometry analysis used ImageJ [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%.

2.4. Western blotting

To quantify both Adonis binding to lymphocytes and the lymphocyte A₂AR 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 A₂AR 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 [36,39,41]. Quantitative variableswere expressed using means ± SEM. Paired t-test was used to compare KᵢD and EC₅₀ values and correlation tests were made between adenosine, B₅₀ and E₅₀ values. All the tests were compared and P-values less than 0.05 were considered 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 value of 18.52 AU ± 8.58 AU.
exceptions, Patient 7 displaying the highest $K_D$ (0.42 $\mu$M) had a 5-fold lower EC$_{50}$ (0.09 $\mu$M) and, conversely, Patient 1 displaying the highest EC$_{50}$ (0.48 $\mu$M) had a 4-fold lower $K_D$ (0.12 $\mu$M) (Fig. 1(D) and Table 1).

3.3. Expression of $A_2AR$

We found various lymphocyte $A_2AR$ expression levels with values ranging from 6.41 to 32.02 AU (pixels of the $A_2AR$ band versus blot background) with a mean value $\pm$ SEM of 14.14 $\pm$ 2.56 AU. Correlation studies were performed to compare lymphocyte $A_2AR$ expressions to APC, $B_{\text{max}}$ and EC$_{50}$. Lymphocyte $A_2AR$ expressions were found to be correlated with both APC (Fig. 2(A)) and $B_{\text{max}}$ (Fig. 2(B)) but not EC$_{50}$ (Fig. 2(C)) values. Once more, we noticed 2 exceptions: Patient 7 displaying the highest EC$_{50}$ (49.89 AU) had low $A_2AR$ level (8.69 AU) while Patient 3 displaying the lowest EC$_{50}$ (16.5 AU) expressed the highest $A_2AR$ level (32.02 AU).

4. Discussion

We found that $B_{\text{max}}$ of Adonis to $A_2AR$ varied from subject to subject whereas $K_D$ values were similar. Various EC$_{50}$ were also found whereas the EC$_{50}$ values were similar. More importantly, the $K_D$ values were found to be statistically not different from the EC$_{50}$ values, suggesting a lack of a reserve of receptors according to the criteria previously defined. In 8 out of the 10 patients tested, the highest cAMP production occurred when all the receptors were occupied by Adonis. Results from 2 patients, however, illustrate opposite situations: Patient 7 exhibiting a $K_D$ which was 5-fold higher than EC$_{50}$, consistent with the situation where a few occupied receptors produce a maximal effect (presence of spare receptors), and Patient 1 exhibited a $K_D$ which was 4-fold lower than EC$_{50}$, indicating a situation where a low effect is obtained while all receptors are occupied. The latter case suggests that many $A_2AR$ of this subject are not functional.

We also performed experiments to address the lymphocyte $A_2AR$ expression using Western blotting. As previously reported in patients with intradialytic hypotension during hemodialysis [43] and spontaneous and head-up-tilt-induced syncope [37,38], we found an overexpression of lymphocyte $A_2AR$ which correlated with the increase in APC and therefore with the maximal level of Adonis binding. This suggests that adenosine and $A_2AR$ are upregulated concomitantly and/or interdependently. More intriguingly, lymphocyte $A_2AR$ expression was generally not correlated with EC$_{max}$ suggesting a non linear relation between the amount of receptors and their overall efficiency. Therefore, adenosine appeared necessary but not sufficient to moderate its immunosuppressive effect via the $A_2AR$. Two patients illustrate opposite situations regarding EC$_{max}$: Patient 7, previously suspected with spare receptors, who expressed a few but highly efficient receptors and, conversely, Patient 3 who expressed high level of receptors exhibiting low overall efficiency, who expressed a few but highly efficient receptors.

Table 1

| Patients | Sex  | Age  | APC (\(\mu\)M) | $B_{\text{max}}$ (AU) | $K_D$ (\(\mu\)M) | EC$_{50}$ (\(\mu\)M) | $A_2AR$ (AU) |
|----------|------|------|--------------|----------------|--------------|----------------|--------------|
| 1        | Female | 62   | 1.55         | 1.22           | 12.37        | 0.13           | 36           |
| 2        | Female | 18   | 0.99         | 32             | 0.11         | 0.06           | 8.61         |
| 3        | Female | 39   | 2.37         | 16.5           | 0.09         | 0.08           | 32.02        |
| 4        | Female | 49   | 1.82         | 28.29          | 0.08         | 0.11           | 12.12        |
| 5        | Female | 74   | 0.77         | 34.62          | 0.33         | 0.36           | 6.41         |
| 6        | Male   | 38   | 2.91         | 41.73          | 0.26         | 0.23           | 17.97        |
| 7        | Male   | 62   | 0.29         | 49.89          | 0.42         | 0.09           | 8.69         |
| 8        | Female | 49   | 0.66         | 31.57          | 0.23         | 0.17           | 7.29         |
| 9        | Female | 52   | 1.01         | 31.57          | 0.08         | 0.11           | 9.24         |
| 10       | Male   | 71   | 0.56         | 36             | 0.13         | 0.06           | 8.61         |
| Mean     | 51.7  | 1.29 | 34.26        | 0.19           | 31.47        | 0.18           | 14.14        |
| ± SEM    | 5.43  | 0.27 | 8.18         | 0.04           | 3.14         | 0.04           | 2.56         |
patients having a specific A2AR gene polymorphism express more receptors [45]. However, the resulting efficiency of the A2AR response can also depend on the functional state of the receptors. The multimerization process of G protein-coupled receptors [46] modulates agonist efficiency when occupancy of a single receptor is needed to obtain a full agonist action. For a recent example, TSH receptor signals via activation of Gs to stimulate cAMP production when TSH binds to one protomer of the TSH receptor homodimer [47]. Therefore, the presence of spare receptors may also refer to the functional state of the multimerized receptors.

Together, these data are consistent with a lack of spare A2AR on lymphocytes as a general rule and their putative presence in some patients appeared to be dependent on the functional state of the targeted receptors. Therefore, the adenosinergic signaling efficiency in lymphocytes likely involves many but not a few A2AR expressed on lymphocytes and this is the amount and functional state of these expressed receptors that determine the maximal level of the lymphocyte response to adenosine.

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Fig. 2. Correlation study between lymphocyte A2AR expression levels and (A) APC, (B) Bmax, and (C) Emax. Results are expressed in μM for adenosine plasma levels and arbitrary units (AU) as defined in Materials and methods for A2AR expression, Bmax and Emax levels. A2AR expressions were measured using Western blotting. Each symbol represents individual mean value. Significant correlation (P = 0.05) for the 10 patients is indicated at right.
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