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Original Research

Effects of salt on A_{2A} adenosine receptors expression and function: *in vitro* approach and pathophysiological perspectives

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Abstract

Salt (sodium chloride) and A_{2A} adenosine receptors ($A_{2A}R$) have both been implicated in blood pressure regulation. While high salt consumption raises blood pressure, $A_{2A}R$ is a key mediator of coronary vasodilation. Although a sodium ion binding pocket has been identified in $A_{2A}R$, the physiological link between salt and $A_{2A}R$ remains poorly investigated. Hereby, we explored how salt modulates its expression and function *in vitro*.

Using Adonis, an IgM mouse monoclonal antibody against $A_{2A}R$ with agonist properties, or CGS21680, an organic agonist, we evaluated the effects of distinct sodium chloride concentrations (low = 120 mM and high = 194 mM, compared to control = 133 mM) on $A_{2A}R$ protein expression, ligand binding affinity (K_D) and function (cAMP production and EC_{50}). We used peripheral blood mononuclear cells, a cellular model mimicking the behavior of $A_{2A}R$ expressed in the cardiovascular system.

After 24h salt supplementation in culture medium, high salt concentration is associated with $A_{2A}R$ increased expression (mean ± SD: +131% ± 25.63 compared with control, $p<0.05$). Upon Adonis activation, K_D and EC_{50} values inversely correlated to salt concentration: the higher salt concentration, the lower K_D and EC_{50} are. High salt concentration is also associated with higher cAMP production upon CGS21680 activation (mean +42%). None of these changes was observed when salt and Adonis were simultaneously added (competition condition).

These results demonstrate that salt promotes $A_{2A}R$ increased expression, binding to Adonis as well as activation. We hypothesize that Adonis binding, potentially due to its large size or to induced conformational changes, allosterically prevents sodium ion from accessing or modulating its binding pocket. This is supported by our competition experiments where simultaneous addition abrogates the salt effect. Our results imply the presence of a novel beneficial compensatory mechanism, via $A_{2A}R$, that responds to elevated blood salt levels and influences blood pressure regulation.

Keywords: Adenosine A_{2A} receptors, Salt, Sodium ion, Blood pressure

Abbreviations: $A_{2A}R$: A_{2A} Adenosine Receptors; GPCRs: G Protein-Coupled Receptors; NaCl: Sodium Chloride; PBMCs: Peripheral Blood Mononuclear Cells; cAMP: Cyclic Adenosine Monophosphate; KD: Dissociation Constant; EC_{50}: Half Maximal Effective Concentration; A.U.: Arbitrary Units; SD: Standard Deviation

Introduction

A_{2A} adenosine receptors ($A_{2A}R$) belong to the superfamily of G protein-coupled receptors (GPCRs), whose activation strongly impacts immune [1], nervous [2] and cardiovascular system [3]. In the cardiovascular area, $A_{2A}R$ stimulation leads to cardiac inotropic effects and vasodilation via cAMP production in target cells, mostly myocytes and endothelial cells in the vascular walls [3], cAMP

production and vasodilation being correlated [4]. $A_{2A}R$ are strongly implicated in blood pressure modulation: high $A_{2A}R$ expression is associated with a drop-in blood pressure [5,6]; conversely, $A_{2A}R$ knockout mice exhibit high systolic blood pressure [7].

Adenosine, a ubiquitous nucleoside mostly derived from ATP dephosphorylation, is the natural ligand of $A_{2A}R$. While high and low adenosine affinities for $A_{2A}R$ have been described [8], it is also established that ligands affinity for $A_{2A}R$ could be regulated in an allosteric manner through small molecules like amiloride or salt (sodium chloride, NaCl) [9–11]. Sodium ions rather than chloride ions are responsible for this allosteric modulation [9,11]. A sodium ion binding pocket has been described in $A_{2A}R$, which is important for regulation of radioligand affinity [10,11]. Interestingly $A_{2A}R$ activation leads to vasodilation and a decrease in blood pressure, while conversely salt excess in blood leads to an increase in blood pressure notably by increasing vascular volume through osmosis mechanism [12–14]. While these effects of salt on one hand and the effects of $A_{2A}R$ activation on the other can be independent, it could be that sodium, apart from its effects on vascular volume, directly impacts $A_{2A}R$ expression and function by binding to an allosteric site.

We have developed an IgM monoclonal antibody (named Adonis) against a linear epitope in the human $A_{2A}R$ second extracellular loop, which also exhibits agonist properties and allows simultaneously measuring affinity (K_D) and function (cAMP production and EC_{50}) [15]. Adonis binds to the orthosteric site with a high affinity (K_D around 0.1-0.2 µM) leading to cAMP production in a dose dependent manner. $A_{2A}R$ protein expression is high in peripheral blood mononuclear cells (PBMCs) and $A_{2A}R$ behavior in these cells reflects the behavior of $A_{2A}R$ expressed in the cardiovascular system [16–20]. Thus, PBMCs represent a good and less invasive model for studying adenosinergic implication in cardiovascular physiopathology.

In order to understand whether salt, and therefore sodium, acts directly on $A_{2A}R$, in this study, by using Adonis and/or an organic agonist, we evaluated the influence of salt on $A_{2A}R$ expression and function in PBMCs.

Material and Methods

Cells culture and NaCl supplementation

PBMCs (Tebubio®, le Perray en Yvelines, France) were cultured in an incubator at 37°C (5% CO_2) in a complete culture medium (RPMI 1640 with 10% heat inactivated Fetal Calf Serum, 100 IU /ml each penicillin/streptomycin and 4 mM glutamine) for 24h. Culture medium was supplemented either with 120 mM (low) or 194 mM (high) NaCl concentrations. 133 mM NaCl concentration, which is similar to human plasma sodium concentration, was defined as control concentration. Any significative difference in viable cells number or size (in µm) or in cell culture medium volume after 24h incubation was observed among the three conditions (data not shown).

For protein expression analysis, after 24h culture cells were centrifuged, and pellets were lysed and used for SDS-PAGE and western blotting analysis, as described below.

To calculate Adonis affinity (K_D), cAMP production and EC_{50}, we used an unconventional technique previously developed in our laboratory [21]. Briefly, after 24h culture, for each sodium chloride concentration, 5 x 10^5 cells were incubated with Adonis, a homemade IgM κ mouse monoclonal antibody directed against a linear epitope on $A_{2A}R$ [15], for 90 min, at increasing concentrations: 0; 0.25; 0.05; 0.1; 0.2; 0.4 or 0.8 µM or with 0.1 µM CGS21680 10 min at 37°C.

For the competition experiments, after 24 h culture in a control (133 mM NaCl) medium, Adonis agonist was incubated alone (no competition) or simultaneously to 194 mM NaCl (competition) to PBMCs for 90 min.

After incubation with the agonists, cells were centrifuged and cells pellets were used to calculate K_D or to measure cAMP production and calculate EC_{50}, as described below.

SDS-PAGE and Western Blotting analysis

Cell pellets (2 × 10^5 cells) were lysed with Laemmli sample buffer and submitted to a standard 12% polyacrylamide gel electrophoresis under reducing conditions followed by transfer onto a PVDF membrane. Membrane was then incubated with Adonis (1 µg/mL), used here as primary antibody, and staining was performed using alkaline phosphatase-labeled anti-mouse antibodies and BCIP / NBT purple liquid substrate (SIGMA® USA). The upper membrane part was incubated with an amido black solution (10% methanol, 10% acetic acid, 0.05% amido black powder) to visualize total protein amounts loaded in each gel lane. For protein expression analysis, the 45-kDa bands corresponding to $A_{2A}R$ were submitted to densitometry analysis using the ImageJ 1.42q software (National Institutes of Health), and values are expressed in arbitrary units (A.U.) as the ratio between pixels generated by $A_{2A}R$ band and pixels generated by the corresponding total proteins bands visualized after amido black staining [19,20]. Values are the mean ± standard deviation (SD) of 5 experiments, and results were expressed as percentage of the control values.

K_D determination

K_D (dissociation constant, which indicates how tightly a ligand binds to a receptor) was defined as ligand (Adonis) concentration (mM) at which 50% of $A_{2A}R$ binding sites were occupied [21–23]. The smaller the K_D, the more tightly bound the ligand is and therefore the higher the affinity between the ligand and the receptor. Western blotting analysis was used to establish the binding curve of Adonis to $A_{2A}R$ on PBMCs and to determine K_D value as previously described [19,24]. Briefly, samples were submitted to a standard 12% electrophoresis under reducing conditions prior to transfer onto a PVDF membrane. These conditions led to the dissociation of Adonis associated with $A_{2A}R$ at the PBMCs surface into its heavy and light chains with only the kappa light chain (25 kDa) being stained here using specific labeled antibodies and substrate. The staining intensity of the light chain was measured using densitometry analysis using the ImageJ 1.42q software (National Institutes of Health) and expressed in A.U. as the ratio between pixels generated by the light chain band and pixels generated by the background signal. K_D values for Adonis binding were estimated using nonlinear regression analysis (Prism software; GraphPad Software), as Adonis concentration at which 50% of the Adonis light chain is released after dissociation. Values are the mean ± SD of 3 experiments.

Measurement of cAMP production and EC_{50}

After PBMCs incubation with the agonists, as described above, cAMP production was measured using cAMP ELISA kit (Cell Biolabs). For EC_{50} (half maximal effective concentration) calculation,

analysis was performed in duplicates and results were expressed as the percentage of the maximal cAMP production. EC_{50} was defined as the concentration of agonist (Adonis) that leads to half maximal stimulation of cAMP production [19,25].

For measurement of cAMP production by CGS21680 data are the means of three experiments and expressed as a percentage of cAMP production variation compared to control condition (133 mM NaCl, C).

Statistical analysis

One-way ANOVA analysis with Tukey's post-hoc test was used for multiple comparison of $A_{2A}R$ expression as well as cAMP production among different conditions. For the competition experiment, an unpaired t-test was used for comparison between "No competition" and "Competition" conditions. A p value <0.05*, <0.001** or <0.0001*** was considered as statistically significant.

Results

Increased $A_{2A}R$ expression in PBMCs cultured with high salt concentration

To understand whether and how salt concentrations modulate $A_{2A}R$ expression, we selected a physiological blood salt concentration, 133 mM NaCl (control condition), a low salt concentration (120 mM NaCl), which is a frequent severe hyponatremia, and an extreme hypernatremia (194 mM NaCl) [26]. PBMCs were cultured in 120 mM or 194 mM NaCl media for 24 h and $A_{2A}R$ protein expression was analyzed by SDS-PAGE and western blotting (**Figure 1**). Variations were expressed as a percentage of the values obtained in control condition. While low NaCl concentration (120 mM) in culture medium did not influence $A_{2A}R$ expression (mean ± SD: 110% ± 11.15 of the control values, p>0.05), high concentration (194 mM) was significantly associated with higher protein expression levels (mean ± SD: +131% ± 25.63 of control values, p<0.05*), which indicates a 31% augmentation.

Ligand affinity for $A_{2A}R$ correlates to salt concentration

To understand whether salt concentration impacts $A_{2A}R$ proprieties, we measured the affinity (K_D) of a homemade antibody Adonis in PBMCs exposed to 120 mM, 133 mM or 194 mM NaCl for 24 h [14,18] (Materials and methods) (**Figure 2** and **Table 1**). K_D is defined as Adonis concentration at which 50% of the $A_{2A}R$ binding sites were occupied and is hereby estimated as Adonis concentration at which 50% of Adonis light chains are released after dissociation. The lower the K_D, the higher ligand affinity with $A_{2A}R$ is.

High concentration in salt (194 mM) is associated with low Adonis K_D value (0.011±0.003 mM, mean -14.5 folds), compared with control conditions (0.16±0.04 mM), while low salt concentration (120 mM) was associated with a higher K_D value (0.3±0.03 mM, mean + 1.9 folds) compared with control conditions. More generally, K_D value inversely correlated to salt concentration, which means that the higher salt concentration, the higher ligand affinity with $A_{2A}R$ is (**Figure 2** and **Table 1**).

Because Adonis is a large IgM [14] and might cover the allosteric sodium binding site, we decided to perform competition experiments between NaCl and Adonis. When Adonis and NaCl (194 mM) are simultaneously added to PBMCs, K_D (0.16 ± 0.02 mM) was 14.5 higher than in no competition conditions (0.011 ± 0.003 mM, remaining approximatively like control condition value (**Figure 3** and **Table 1**). Finally, regardless of salt concentration, no change was observed during competitive experiments between Adonis and CGS21680 compared to no competition conditions (results not shown).

High salt concentration decreases Adonis EC_{50}

To understand whether salt concentration impacts $A_{2A}R$ function, we measured Adonis cAMP production and EC_{50} in PBMCs exposed to 120 mM, 133 mM or 194 mM NaCl for 24h (Material and methods) (**Figure 4** and **Table 1**). EC_{50} was defined as the concentration of agonist (Adonis) that leads to half maximal stimulation of cAMP production. The lower the EC_{50}, the higher ligand capacity to activate $A_{2A}R$ is.

High salt concentration (194 mM) is associated with low EC_{50} value (0.08±0.01 mM, mean -2.5 folds lower), compared with control conditions (0.20±0.01 mM), while low concentration (120

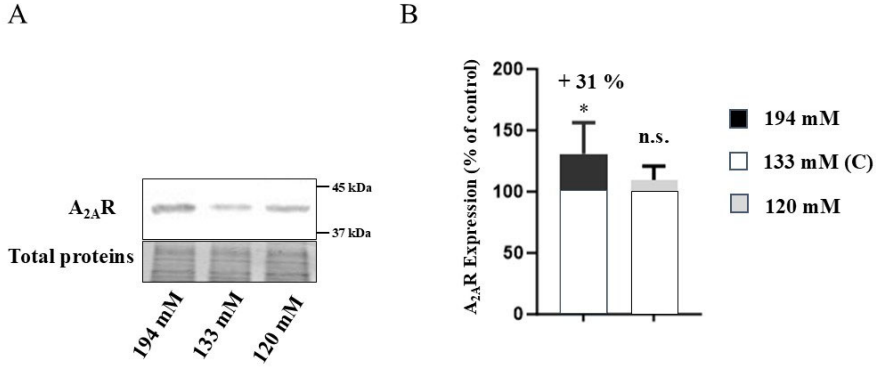

Figure 1. Increased $A_{2A}R$ expression in PBMCs cultured with high salt concentration. PBMCs were cultured for 24h in 194 mM, 133 mM or 120 mM sodium chloride concentrations. $A_{2A}R$ protein (45kDa band) expression was analyzed by SDS-PAGE and western blotting (**A**), then quantified by Image J in arbitrary units (normalized on total proteins amount visualized by Amido black staining), and then expressed as percentage (%) of the $A_{2A}R$ expression values obtained in the control condition (133 mM). (**B**) Data are presented as mean ± SD (N = 5). One-way ANOVA analysis with Tukey's post-hoc test. A p value <0.05*, <0.001** or <0.0001*** was considered as statistically significant. n.s.: not significant, p>0.05 compared to control.

Table 1. Summary of salt effects on affinity (K_D) and EC_{50} values, using Adonis antibody as agonist (see Material and methods).

	Salt (194 mM)	Salt (133 mM)	Salt (120 mM)	Salt (194 mM) Competition conditions
K_D (µM)	0.011 ± 0.003	0.16 ± 0.04	0.3 ± 0.03	0.16 ± 0.02
EC_{50} (µM)	0.08 ± 0.01	0.20 ± 0.01	0.38 ± 0.04	0.26 ± 0.11

Data are presented as mean of triplicated (K_D) or duplicated (EC_{50}) experiments ± SD.

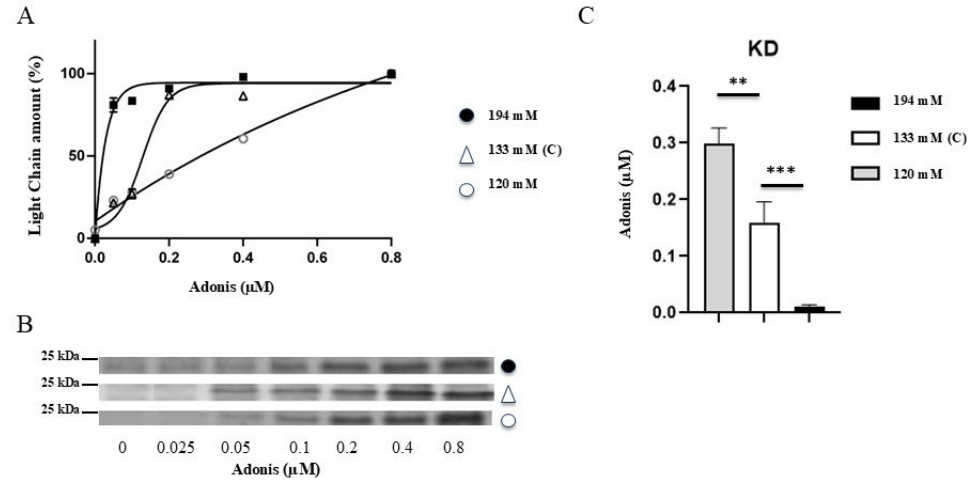

Figure 2. **Effects of salt concentration on Adonis affinity (K_D).** PBMCs were exposed to three sodium concentrations (120 mM, 133 mM and 194 mM) for 24 h and K_D was measured and quantified as described in Material and methods. (**A**) Data are expressed as a percentage of the total amount of Adonis light chain binding and presented as mean of triplicated experiments (see also **Table 1**). (**B**) Adonis light chain bound to PBMCs visualized by Western blot analysis. (**C**) K_D values are presented as mean ± SD. One-way ANOVA analysis with Tukey's post-hoc test. A p value <0.05*, <0.001** or <0.0001*** was considered as statistically significant.

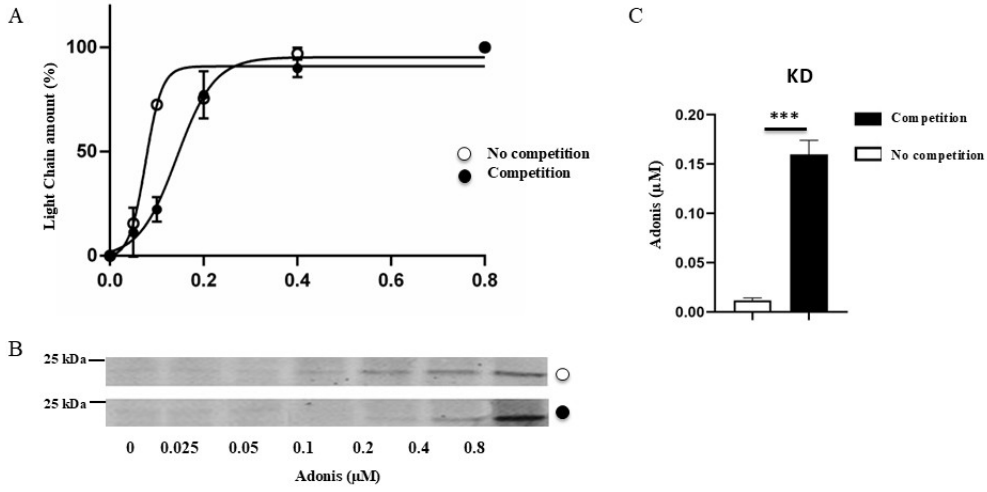

Figure 3. Effects of competition between Adonis and high salt concentration on affinity (K_D). PBMCs were incubated with Adonis in the absence (no competition, dark round) or presence (competition, white round) of 194 mM sodium chloride concentration. K_D was measured and quantified as described in Material and methods. (**A**) Data are expressed as a percentage of the total amount of Adonis light chain binding and presented as mean of duplicated experiments (see also **Table 1**). (**B**) Adonis light chain bound to PBMCs visualized by Western blot analysis. (**C**) K_D values are presented as mean ± SD. An unpaired t-test was used for comparison. A p value <0.05*, <0.001** or <0.0001*** was considered as statistically significant.

mM) was associated with a highest EC_{50} value (0.38±0.04 mM, mean + 1.8 folds higher) compared with control conditions. More generally, EC_{50} value inversely correlates to salt concentration, which means that the higher salt concentration, the higher ligand capacity to activate $A_{2A}R$ is. In competition conditions, EC_{50} was higher (0.26±0.11 mM, mean 3.25 folds higher) compared to conditions without competition and similar to the control values (133 mM) (**Table 1**).

In summary, in non-competitive conditions, salt concentration is associated with a decrease in K_D and EC_{50} values in a dose-depending manner. When salt and Adonis were added simultaneously in culture medium (competitive condition), the presence of Adonis eliminates all the effects observed upon salt preincubation.

High salt concentration increases CGS21680 dependent cAMP production

Additionally, we measured cAMP production by CG21680 agonist in PBMCs exposed to 120 mM, 133 mM or 194 mM NaCl for 24 h by ELISA (Material and methods). While low salt (120 mM) concentration was not associated with significant change in cAMP production after CGS21680 incubation (83.7% ±13.9), compared with control conditions, high sodium concentration (194 mM) was associated with a higher cAMP production by CGS21680 (142.0% ± 15.6, mean + 42%) compared with 133 mM (**Figure 5**).

Discussion

The main results of this study are that: 1) high salt concentration is associated with a higher protein expression of $A_{2A}R$ compared with control salt concentration and 2) salt decreases K_D and EC_{50} values (evaluated with Adonis a monoclonal antibody with agonist properties) in a dose dependent manner. High salt concentration also increased cAMP production by the organic agonist CGS21680. These results indicate that salt promotes $A_{2A}R$ agonist binding and cAMP production.

It is now established that orthosteric binding pocket of a ligand represents just one among several sites for possible signal modulation

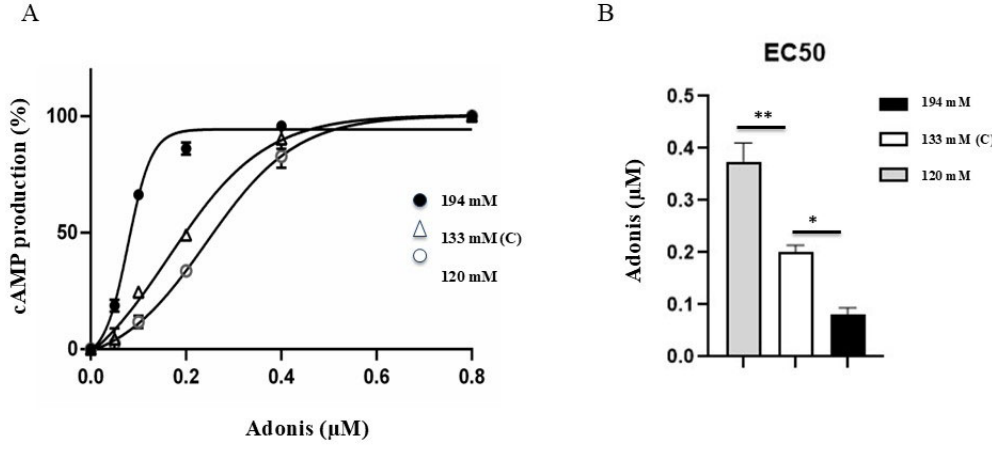

Figure 4. **Effects of salt concentration on Adonis cAMP production and EC_{50}.** PBMCs were exposed to three concentrations (120 mM, 133 mM and 194 mM) for 24h and then incubated with Adonis. cAMP production was measured and quantified as described in Material and methods. (**A**) Data are presented as mean of duplicated experiments and expressed as a percentage of the total cAMP production (see also **Table 1**). (**B**) EC_{50} values are presented as mean ± SD. One-way ANOVA analysis with Tukey's post-hoc test. A p value <0.05*, <0.001** or <0.0001*** was considered as statistically significant.

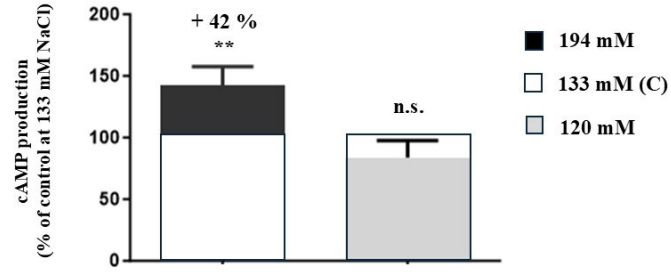

Figure 5. **High sodium concentration (194 mM) was associated with a higher cAMP production by CGS21680.** Effects of sodium chloride concentration on cAMP production in PBMCs incubated with CGS21680 (Material and methods). Data are the means of three experiments and expressed as a percentage of cAMP production variation compared to control condition (133 mM NaCl, C). One-way ANOVA analysis with Tukey's post-hoc test. A p value <0.05*, <0.001** or <0.0001*** was considered as statistically significant.

in GPCRs and that allosteric binding pocket not only impacts ligand affinity but also plays a major role in receptor signaling [27–29]. Thus, molecules different from ligand can modulate binding of natural ligand to the orthosteric site. It was shown that small molecules like amiloride, ions or lipids can function as allosteric modulators of GPCR [9–11,27,29,30]. While chloride ion did not show any modulatory function [9], the allosteric effect of sodium ion has been previously described in A_1R [31] and $A_{2A}R$ structural models [9–11,32]. Modeling of sodium binding to $A_{2A}R$ suggests that sodium ion stabilizes $A_{2A}R$ in its inactive conformation and destabilizes activation-related movements and agonist binding [32]. It is admitted that sodium ion induces an increase in radioligand antagonist binding but abrogates agonist binding in a dose dependent manner [9–11]. Nonetheless, these results were mainly obtained in structural and computational studies, where molecular simulations were combined with biophysical and biochemical experiments performed mainly by using membranes from HEK293T cells transfected with wild type or mutated $A_{2A}R$. Additionally, salt concentrations used in these assays were rather much higher or lower than physiological levels [9–11,32]. Hereby, we have used a physiological cell culture model (not transfected PBMCs, expressing $A_{2A}R$ protein), incubated with salt concentrations reflecting physiological or pathological natremia. In contrast to those previous studies, we found that cells supplementation with high salt concentration is associated with an increased affinity (K_D decrease) and a decrease in EC_{50} by agonists in a dose-dependent manner, which suggests that sodium ions promote $A_{2A}R$ activation. While we cannot completely exclude that the observed effects of NaCl concentration on $A_{2A}R$ might depend on osmotic variations, any significative difference in viable cells number or size (in µm) or in cell culture medium volume after 24h incubation was observed among the three salt conditions (data not shown). Therefore, currently, we are more confident on the direct allosteric effect of sodium on $A_{2A}R$ modulation.

In competition conditions, when Adonis and sodium ion are added simultaneously in culture medium, any change on K_D and EC_{50} was observed compared to control (no competition) condition. We hypothesize that Adonis (a large IgM) binding to the receptor prevents sodium integration in its pocket, because it either induces $A_{2A}R$ conformational changes, as described for other GPCRs [33,34] or for its size it extends beyond the orthostatic site covering the allosteric sodium pocket on the second trans-membrane helix [15,35]. Adonis, for the same reasons, also prevents CGS21680 from being properly fixed on its site, which could explain the absence of additive effects on cAMP production when CGS and Adonis are both added to the culture medium (data not shown). Whether it is Adonis large size or its effect on $A_{2A}R$ conformation to perturb sodium binding needs further investigation. Moreover, although our antibody seems to behave like an organic agonist, particularly in terms of orthostatic binding site and cAMP production, the interactions between Adonis and $A_{2A}R$ receptor may be much more complex than with small organic molecules, such the natural ligand adenosine.

Pathophysiologic perspectives

The relationship between salt, blood pressure, and adenosinergic system has been studied in the context of renal physiology. It was shown that adenosine impacts glomerular filtration and, in this context, may act on blood pressure through diuresis modulation [36]. Infusion of adenosine into the renal artery also produces diuresis in rats [37] and promotes salt excretion [36].

In a more general perspective, it is well-established that excess salt increases blood pressure by a whole series of mechanisms. The first of these is the drawing of water into the vessels, resulting in hypervolemia. But salt also stimulates the sympathetic system, resulting in vasoconstriction [38]. Salt elevation in the cerebrospinal fluid also leads to activation of angiotensin II and ouabain-dependent sodium pump, leading to the increase in blood pressure [38,39]. Although excess salt has long been known to increase arterial tension [40], the relationship between salt and cardiovascular complications is not clear [41]. It is likely that there are also compensatory mechanisms for the rise in arterial pressure induced by excess salt. Increase of $A_{2A}R$ expression and function (via increase of affinity for adenosine) could be one of such beneficial compensatory mechanisms.

Conclusions

Our findings point to the potential role of $A_{2A}R$/sodium ion direct interaction in blood pressure regulation and suggest the presence of a compensatory mechanism for the increase in arterial pressure induced by excess salt.

Conflicts of Interest

The authors declare that they have no conflicts of Interest.

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Not applicable.

Authors' Contributions

EOF and SA: Data acquisition, analysis, interpretation; GC, MM, BAP, KN, CC, CMC, LS: Data acquisition, interpretation; DJ, FJ and RJ: revision work; GR and MG: Conception, design, data analysis and interpretation, manuscript editing. All authors read and approved the final manuscript.

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