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A_{2A} adenosine receptor ligand binding and signaling is allosterically modulated by adenosine deaminase

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Running title: Adenosine deaminase as allosteric modulator of A_{2A} adenosine receptors

SYNOPSIS

Adenosine A_{2A} receptors (A_{2A}R) are highly enriched in the striatum, which is the main motor control CNS area. Bioluminescence resonance energy transfer (BRET) assays showed that A_{2A}R homomers may act as cell surface adenosine deaminase (ADA; EC 3.5.4.4) binding proteins. ADA binding affected the quaternary structure of A_{2A}R present on the cell surface. ADA binding to adenosine A_{2A} receptors increased both, agonist and antagonist affinity on ligand binding to striatal membranes where these proteins are co-expressed. ADA also increased receptor-mediated ERK 1/2 phosphorylation. Collectively, the results showed that ADA, apart from regulating the concentration of extracellular adenosine, may behave as an allosteric modulator that markedly enhances ligand affinity and receptor function. This powerful regulation may have implications for the physiology and pharmacology of neuronal adenosine A_{2A} receptors.

KEYWORDS

G-protein coupled receptors, receptor kinetics, protein-protein interaction, adenosine deaminase, adenosine receptors, allosteric interactions

ABBREVIATIONS

ADA: adenosine deaminase; ARs: adenosine receptors; BRET: bioluminescence resonance energy transfer; CHO: chinese hamster ovary cells; ERK: extracellular signal-regulated kinase; GABA: γ -aminobutyric acid; GPCRs: G protein-coupled receptors; HEK: human embryonic kidney cells; [³H]CGS 21680: tritium labeled 4-[2-[[6-Amino-9-(*N*-ethyl- β -D-ribofuranuronamidosyl)-9*H*-purin-2-yl]amino]ethyl]benzenepropanoic acid; [³H]ZM 241385: tritium labeled 4-(2-[7-Amino-2-(2-furyl)[1,2,4]triazolo[2,3-*a*][1,3,5]triazin-5-ylamino]ethyl)phenol; NMDA: N-methyl-D-aspartic acid; R-luc: R-luciferase; SCID: severe combined immunodeficiency; YFP: yellow fluorescence protein.

INTRODUCTION

Self-association of proteins to form dimers and higher order oligomers and/or interaction with other proteins are key factors in cell signalling [1-3]. A paradigmatic example are adenosine receptors. The nucleoside adenosine exerts a modulatory action in many areas of the central nervous system (CNS) via its four G-protein-coupled receptor (GPCRs) subtypes: the adenosine A₁ (A₁Rs) and A₃ (A₃Rs) receptors that are negatively coupled to the adenylate cyclase, and the adenosine A_{2A} (A_{2A}Rs) and A_{2B} (A_{2B}Rs) receptors that mediate the stimulation of adenylate cyclase activity [4]. Along the plasma membrane (horizontal plane) A₁Rs and A_{2A}Rs may form homooligomers [5-7] and heteromers with other receptors [8-11] and the oligomerization generate new and unique biochemical and functional characteristics by modulating the binding properties, G-protein coupling and receptor trafficking [3, 12, 13]. Across the membrane (vertical to the plane of the membrane), A₁Rs interact with intracellular proteins that are not directly involved in signalling cascade, as the heat-shock cognate protein hsc73, and this direct interaction is relevant for receptor function [14]. Also across the membrane, both A₁Rs and A_{2B}Rs interact with a protein that has an extracellular topology, the adenosine deaminase (ADA) [15-18].

ADA is an enzyme involved in the purine metabolism that catalyzes the hydrolytic deamination of adenosine and 2'-deoxyadenosine to inosine or 2'-deoxyinosine and ammonia. Congenital defect of ADA leads to severe combined immunodeficiency (SCID), which is characterized by the absence of functional T and B lymphocytes in affected individuals [19, 20]. Neurological abnormalities, which are less life threatening than immunological abnormalities, have been also described in a portion of patients [21]. Neurological alterations may be secondary to infections or may be due to the accumulation of adenosine and derivatives in brain. Although the location of ADA is mainly cytosolic, it has been found on the cell surface of many cell types including neurons [22]; therefore, it can be considered as an ecto-enzyme [19]. Since ADA is a peripheral membrane protein it needs of integral membrane proteins to be anchored to the membrane. Apart from A₁Rs and A_{2B}Rs another class of ecto-ADA binding protein is CD26, a multifunctional transmembrane glycoprotein, acting as a receptor and a proteolytic enzyme [23]. It has been shown that ADA anchored to the dendritic cells surface probably by the A_{2B}R, binds to CD26 expressed on the surface of T cells triggering co-stimulation and enabling enhanced immune response [24-26].

We have also demonstrated that binding of enzymatically active or inactive ADA to A_{2B}R increases its affinity and signalling by a protein-protein interaction [17]. In the case of A₁Rs, the ADA/A₁R interaction is very relevant since the enzyme potentiates signal transduction and modulates the desensitization of A₁Rs [15, 18, 27]. Despite the well established positive modulation exerted by ADA on A₁Rs and A_{2B}Rs, it is not known if the enzyme is able to modulate the A_{2A}R subtype. There is currently a major interest in the ability of central A_{2A}Rs to control synaptic plasticity at glutamatergic synapses due to a combined ability of these receptors to facilitate the release of glutamate and the activation of NMDA; furthermore, A_{2A}Rs also control glia function and brain metabolic adaptation, and are important in controlling the demise of neurodegeneration [28]. In the present paper we report the molecular interaction between ADA and A_{2A}R that results in ADA-induced conformational changes in the quaternary structure of A_{2A}Rs homodimers and in the pharmacological and functional characteristics of brain striatal A_{2A}Rs. A fine tune regulation exerted by ADA likely has important implications for the physiology and pharmacology of neuronal A_{2A}Rs.

EXPERIMENTAL

Fusion Proteins and Expression Vectors

The human cDNA for the adenosine A_{2A} or GABA_{B2} receptors cloned in pcDNA3.1 were amplified (removing stop codons) using sense and antisense primers harboring either unique *EcoRI* and *KpnI* sites. The fragments were then subcloned to be in-frame with Rluc into the *EcoRI* and *KpnI* restriction site of an Rluc-expressing vector (pRluc-N1; PerkinElmer, Wellesley, MA), or into the *EcoRI* and *KpnI* or *BamHI* restriction site of the variant of GFP (EYFP-N3; enhanced yellow variant of GFP; Clontech, Heidelberg, Germany), to give the plasmids that express A_{2A} or GABA_{B2} receptors fused to Rluc or YFP on the C-terminal end of the receptor (A_{2A}R-Rluc, A_{2A}R-YFP or GABA_{B2}R-Rluc). As previously reported [9, 11], when analyzed by confocal microscopy, it was observed that all fusion proteins showed similar membrane distribution than naïve receptors and fusion of *Rluc* and YFP to A_{2A}R receptors did not modify receptor function as determined by cAMP assays.

Transient Transfection

HEK-293T cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco Paisley, Scotland, UK) supplemented with 2 mM L-glutamine, 100 U/ml penicillin/streptomycin, and 5% (v/v) heat inactivated Foetal Bovine Serum (FBS) (all supplements were from Invitrogen, Paisley, Scotland, UK). HEK-293T growing in 6-well dishes were transiently transfected with the corresponding fusion protein cDNA by the PEI (PolyEthylenImine, Sigma, Steinheim, Germany) method. Cells were incubated (4 h) with the corresponding cDNA together with PEI (5.47 mM in nitrogen residues) and 150 mM NaCl in a serum-starved medium. After 4 hours, the medium was changed to a fresh complete culture medium. Forty-eight hours after transfection, cells were washed twice in quick succession in HBSS with 10 mM glucose, detached, and resuspended in the same buffer containing 1mM EDTA. To control the cell number, sample protein concentration was determined using the bicinchoninic acid method (Pierce Chemical Co., Rockford, IL, USA) using bovine serum albumin dilutions as standards.

Generation of a CHO cell clone expressing A_{2A}Rs

CHO cell were maintained at 37°C in an atmosphere of 5% CO₂ in α -MEM medium without nucleosides (Invitrogen, Paisley, Scotland, UK), containing 10% fetal calf serum, 50 μ g/mL penicillin, 50 μ g/mL streptomycin, and 2 mM L-glutamine (300 μ g/mL). CHO cells were transfected with the cDNA corresponding to human A_{2A}R, cloned into a pcDNA3.1/Hygro vector with a hygromycin resistance gene, by lipofectamine (InvitrogenTM, Carlsbad, USA) method following the instructions of the supplier. At one day of transfection, the selection antibiotic was added at a concentration that was previously determined by a selection antibiotic test. The antibiotic resistant clones were isolated and cultured in 6-well plates in the presence of the selection antibiotic. After an appropriate number of days/passes a stable line expressing 6 ± 1 pmol/mg of protein with an affinity constant for the A_{2A}R antagonist ZM 241385 of 1 ± 0.3 nM, was selected and cultured in the presence of hygromycin (300 μ g/ml).

Bioluminescence Resonance Energy Transfer (BRET)

HEK-293T cells were co-transfected with 0.15 μ g of cDNA corresponding to A_{2A}R-Rluc, acting as a BRET donor and increasing amounts of cDNA corresponding to A_{2A}R-

YFP (0.8 to 3 μg cDNA), acting as a BRET acceptor. As negative control, HEK-293T cells were co-transfected with 0.15 μg of $A_{2A}R$ -Rluc and increasing amounts of cDNAs corresponding to $GABA_{B2}$ -YFP receptor (0.3 to 3 μg cDNA). After 48 h of transfection cell suspension (20 μg of protein) was dispensed in duplicates into 96-well black microplates with a transparent bottom (Porvair, King's Lynn, UK), and the fluorescence was measured using a Mithras LB940 fluorescence-luminescence detector (Berthold, Bad Wildbad, Germany) with an excitation filter of 485 nm and an emission filter of 535 nm. For BRET measurement, 20 μg of cell suspension were distributed in duplicates into 96-well white opaque microplates (Porvair), and coelenterazine H (Molecular Probes Europe, Leiden, The Netherlands) was added at a final concentration of 5 $\mu\text{mol}\cdot\text{L}^{-1}$. After 1 min the readings were collected in a Mithras LB 940 that allows the integration of the signals detected in the short-wavelength filter at 485 nm (440-500 nm) and the long-wavelength filter at 530 nm (510-590 nm). The same samples were incubated for 10 min, and the luminescence was measured to quantify the donor. The BRET ratio is defined as [(emission at 510-590)/(emission at 440-500)]-Cf, where Cf corresponds to (emission at 510-590)/(emission 440/500) for the A_{2A} -Rluc construct expressed alone in the same experiment. Curves were fitted to a non-linear regression equation, assuming a single phase with Graph-Pad Prism software (San Diego, CA, USA).

Immunostaining

Wild type CHO cells and $A_{2A}R$ expressing CHO cells, grown on glass coverslips, were washed with phosphate-buffered saline and fixed with 2% paraformaldehyde, 60 mM sucrose, pH 7.4 for 15 min at room temperature. Cells were washed twice with phosphate-buffered saline containing 15 mM glycine and treated with 1% BSA, 20 mM glycine and 0.05% NaN_3 for 20 min before the addition of the antibodies. Then, cells were labelled for 45 min either with 100 $\mu\text{g}/\text{ml}$ of the anti- $A_{2A}R$ antibody [14, 29] or 50 $\mu\text{g}/\text{ml}$ of the anti-ADA antibody [30], both conjugated with Fluorescein-5-isothiocyanate (FITC) as previously described [14]. Cells were washed with phosphate-buffered saline containing 1% BSA, 20 mM glycine and 0.05% NaN_3 , and placed in coverslips for the subsequent fluorescence microscopy analysis in a Leica TCS 4D confocal scanning laser microscope (Leica Lasertechnik GmbH, Heidelberg, Germany).

Brain striatal membranes preparation and protein determination

Membrane suspensions from sheep brain striatum were prepared as described previously [31]. Tissue was disrupted with a Polytron homogenizer (PTA 20 TS rotor, setting 3; Kinematica, Basel, Switzerland) for three 5 s-periods in 10 volumes of 50 mM Tris-HCl buffer, pH 7.4, containing a protease inhibitor cocktail (Sigma, 1/1000). After eliminating cell debris by centrifugation at 1,000 g for 10 min, membranes were obtained by centrifugation at 105,000 g (40 min, 4°C) and the pellet was resuspended and recentrifuged under the same conditions. The pellet was stored at -80°C and was washed once more as described above and resuspended in 50 mM Tris-HCl buffer for immediate use. Protein was quantified by the bicinchoninic acid method (Pierce Chemical Co., Rockford, IL, USA) using bovine serum albumin dilutions as standard.

Enzyme activity of ADA and ADA inhibition by Hg^{2+}

Bovine ADA (Roche, Basel, Switzerland) enzyme activity was determined at 25°C with 0.1 mM adenosine as substrate in 50 mM Tris-HCl buffer, pH 7.4. The decrease in the absorbance at 265 nm ($\Delta\epsilon = 7,800 \text{ M}^{-1}\text{cm}^{-1}$) was monitored in an Ultrospec 3300 *pro* spectrophotometer (Biochrom Ltd, Cambridge, UK); 1 ml cuvettes with a 1 cm light

path length were used. Hg²⁺-inactivation of bovine ADA was performed by a pre-incubation (2h), of 15 U/ml of desalted ADA with 100µM HgCl₂ and removal of free Hg²⁺ by gel filtration as described previously [16]. No residual activity was found after 4h incubation with 0.1 mM adenosine and a high excess (10 µg/ml) of inhibited enzyme in the conditions described above.

Radioligand binding experiments

ADA dose-dependent curves were obtained by incubating (2 h) sheep brain striatal membrane suspensions (0.3 mg of protein/ml) with the indicated concentration of A_{2A}R agonist [³H]CGS 21680 (42.7 Ci/mmol; Perkin Elmer, Boston, MA, USA) or A_{2A}R antagonist [³H]ZM 241385 (27 Ci/mmol; American Radiolabelled Chemicals, St. Louis, MO, USA) in the presence or the absence of the indicated amounts of desalted bovine ADA at 25°C in 50 mM Tris-HCl buffer, pH 7.4, containing 10 mM MgCl₂.

Saturation experiments were performed by incubating striatal membrane suspensions (0.3 mg of protein/ml) with increasing concentrations of the adenosine A_{2A}R antagonist [³H]ZM 241385 (triplicates of 10 different concentrations, from 0.1 to 27 nM), at 25°C in 50 mM Tris-HCl buffer, pH 7.4, containing 10 mM MgCl₂, in the absence or the presence of 0.2 I.U./ml (1 µg/ml) of ADA.

Competition experiments were performed by incubating striatal membrane suspensions (0.3 mg of protein/ml) with a constant amount of [³H]CGS 21680 or [³H]ZM 241385 and increasing concentrations of CGS 21680 (triplicates of 10 different concentrations from 1 nM to 10 µM; Tocris, Ellisville, MO, USA) or ZM 241385 (triplicates of 11 different concentrations, from 0.01 nM to 10 µM; Tocris) in the absence or in the presence of 0.2 I.U./ml (1 µg/ml) of desalted ADA at 25°C in 50 mM Tris-HCl buffer, pH 7.4, containing 10 mM MgCl₂, providing sufficient time to achieve equilibrium for the lowest radioligand concentration (5 h). In all experiments nonspecific binding was determined in the presence of 10 µM CGS 21680 or 10 µM ZM 241385 and confirmed that the value was the same as calculated by extrapolation of the competition curves. Free and membrane bound ligand were separated by rapid filtration of 500 µl aliquots in a cell harvester (Brandel, Gaithersburg, MD, USA) through Whatman GF/C filters embedded in 0.3% polyethylenimine that were subsequently washed for 5 s with 5 ml of ice-cold Tris-HCl buffer. The filters were incubated with 10 ml of Ecoscint H scintillation cocktail (National Diagnostics, Atlanta, GA, USA) overnight at room temperature and radioactivity counts were determined using a Tri-Carb 1600 scintillation counter (PerkinElmer, Boston, MO, USA) with an efficiency of 62% [14].

Binding data analysis

Since A_{2A}R are expressed as dimers or higher order oligomers [6, 13], radioligand competition curves were analyzed by nonlinear regression using the commercial Grafit curve-fitting software (Erithacus Software, Surrey, UK), by fitting the specific binding data to the mechanistic two-state dimer receptor model [32, 33]. This model considers a homodimer as the minimal structural unit of the receptor. To calculate the macroscopic equilibrium dissociation constants from saturation binding experiments the following equation previously deduced [34] was considered:

$$A_{\text{bound}} = (K_{\text{DA}2} A + 2 A^2) R_{\text{T}} / (K_{\text{DA}1} K_{\text{DA}2} + K_{\text{DA}2} A + A^2) \quad \text{eq. 1}$$

where A represents the free radioligand (the adenosine A_{2A} receptor antagonist [³H]ZM 241385) concentration, R_T is the total amount of receptor dimers, and K_{DA1} and K_{DA2}

are the macroscopic dissociation constants describing the binding of the first and the second radioligand molecule to the dimeric receptor.

When binding of A to the dimer is non-cooperative, $K_{DA2}/K_{DA1} = 4$ [see 32, 33, for details] and, therefore, K_{DA1} is enough to characterize the binding. In this case, the above equation can be reduced to:

$$A_{\text{bound}} = 2A R_T / (2 K_{DA1} + A) \quad \text{eq. 2}$$

To calculate the macroscopic equilibrium dissociation constants from competition binding experiments the following equation previously deduced [34, 35] was considered:

$$A_{\text{total bound}} = (K_{DA2} A + 2A^2 + K_{DA2} A B / K_{DAB}) R_T / (K_{DA1} K_{DA2} + K_{DA2} A + A^2 + K_{DA2} A B / K_{DAB} + K_{DA1} K_{DA2} B / K_{DB1} + K_{DA1} K_{DA2} B^2 / (K_{DB1} K_{DB2})) + A_{\text{non-specific bound}} \quad \text{eq. 3}$$

here A represents free radioligand (the adenosine A_{2A} receptor agonist [^3H]CGS 21680 or the adenosine A_{2A} receptor antagonist [^3H]ZM 241385) concentration, B represents the assayed competing compound (CGS 21680 or ZM 241385) concentration, and K_{DB1} and K_{DB2} are, respectively, the macroscopic equilibrium dissociation constants of the first and second binding of B; K_{DAB} is the hybrid equilibrium radioligand/competitor dissociation constant, which is the dissociation constant of B binding to a receptor dimer semi-occupied by A.

Binding to GPCRs can displays negative cooperativity and in these circumstances $K_{D2}/K_{D1} > 4$. On the other hand, for positive cooperativity, $K_{D2}/K_{D1} < 4$ [34]. To measure the degree of cooperativity, the two-state dimer receptor model also introduces a cooperativity index (D_C). The dimer cooperativity index for the radioligand A ([^3H]ZM 241385) or the competing ligand B (CGS 21680 or ZM 241385) was calculated as [13, 34, 35]:

$$D_{CA} = \log (4K_{DA1} / K_{DA2}); D_{CB} = \log (4K_{DB1} / K_{DB2})$$

D_C measures the affinity modifications occurring when a protomer senses the binding of the same ligand molecule to the partner protomer in a dimer. The way the index is defined is such that its value is "0" for noncooperative binding, positive values of D_C indicate positive cooperativity, whereas negative values imply negative cooperativity [13, 34, 35].

In the experimental conditions when both the radioligand A ([^3H]CGS 21680 or [^3H]ZM 241385) and the competitor B (CGS 21680 or ZM 241385) show non-cooperativity ($D_C = 0$), it results that $K_{DA2} = 4K_{DA1}$ and $K_{DB2} = 4K_{DB1}$, and eq. (3) was simplified to:

$$A_{\text{total bound}} = (4K_{DA1} A + 2A^2 + 4K_{DA1} A B / K_{DAB}) R_T / (4K_{DA1}^2 + 4K_{DA1} A + A^2 + 4K_{DA1} A B / K_{DAB} + 4K_{DA1}^2 B / K_{DB1} + K_{DA1}^2 B^2 / K_{DB1}^2) + A_{\text{non-specific bound}} \quad \text{eq. 4}$$

When both the radioligand A ($[^3\text{H}]\text{CGS 21680}$ or $[^3\text{H}]\text{ZM 241385}$) and the competitor B are the same compound and the binding is non-cooperative, eq. (4) simplifies to:

$$A_{\text{total bound}} = (4K_{\text{DA1}} A + 2A^2 + A B) R_{\text{T}} / (4K_{\text{DA1}}^2 + 4K_{\text{DA1}} A + A^2 + A B + 4K_{\text{DA1}} B + B^2) + A_{\text{non-specific bound}} \quad \text{eq. 5}$$

Goodness of fit was tested according to reduced χ^2 value given by the nonlinear regression program. The test of significance for two different population variances was based upon the F-distribution (see 32 for details). Using this *F* test, a probability greater than 95% ($p < 0.05$) was considered the criterion to select a more complex equation to fit binding data over the simplest one. In all cases, a probability of less than 70% ($p > 0.30$) resulted when one equation to fit binding data was not significantly better than the other. Results are given as parameter values \pm S.E.M. of three-four independent experiments.

ERK phosphorylation assay

$A_{2A}\text{Rs}$ expressing CHO cells were cultured in serum-free medium for 16 h before the addition of any agent. Cells were treated (1h, 37°C) with medium or the indicated concentration of ADA before the addition of the $A_{2A}\text{R}$ agonist CGS 21680 for a further incubation of 5 min. Cell were washed with ice-cold phosphate-buffered saline and lysed by the addition of 500 μL of ice-cold lysis buffer (50 mM Tris-HCl pH 7.4, 50 mM NaF, 150 mM NaCl, 45 mM β -glycerophosphate, 1% Triton X-100, 20 μM phenylarsine oxide, 0.4 mM NaVO_4 and protease inhibitor cocktail). Cell debris were removed by centrifugation at 13,000 g for 5 min at 4°C and the protein was quantified by the bicinchoninic acid method using bovine serum albumin dilutions as standards. To determine the level of ERK1/2 phosphorylation, equivalent amounts of protein (15 μg) were separated by electrophoresis on a denaturing 10% SDS-polyacrylamide gel and transferred onto PVDF-FL membranes. Odyssey blocking buffer (LI-COR Biosciences, Lincoln, Nebraska, USA) was then added and membranes were rocked for 90 min. Membranes were then probed with a mixture of a mouse anti-phospho-ERK 1/2 antibody (1:2500, Sigma, Steinheim, Germany) and rabbit anti-ERK 1/2 antibody (1:40000, Sigma) for 2-3 h. Bands were visualized by the addition of a mixture of IRDye 800 (anti-mouse) antibody (1:10000, Sigma) and IRDye 680 (anti-rabbit) antibody (1:10000, Sigma) for 1 h and scanned by the Odyssey infrared scanner (LI-COR Biosciences, Lincoln, Nebraska, USA). Bands densities were quantified using the scanner software and exported to Excel (Microsoft, Redmond, WA, U.S.A). The level of phosphorylated ERK1/2 isoforms was normalized for differences in loading using the total ERK protein band intensities.

RESULTS

ADA was anchored to the cell surface of A_{2A}R-expressing cells

To investigate a potential direct interaction of ADA and A_{2A}Rs, wild type CHO cells and a CHO-A_{2A}R clone were selected since CHO cells do not constitutively express adenosine receptors and since rodent CD26 endogenously expressed in CHO cells do not interact with ADA [36]. Parental CHO cells did not express A_{2A}Rs since they could not be labelled using a specific anti-A_{2A}R antibody (Fig. 1b). The CHO-A_{2A}R clone showed a marked staining for A_{2A}R (Fig. 1a). ADA, which was detected in the cytoplasm using permeabilized CHO cells (results not shown), did not appear at the cell surface of parental CHO cells (Fig. 1d). However, cell surface ADA was detected in CHO-A_{2A}R cells (Fig. 1c) indicating that the ADA released to the cell culture may bind to the cell surface only in cells expressing A_{2A}Rs. These results indicate that the cell surface A_{2A}R behaved as an ADA anchoring protein.

ADA binding affected the quaternary structure of A_{2A}Rs

To investigate the consequences of the ADA-A_{2A}Rs interaction, and taking into consideration that A_{2A}R are expressed as dimers or higher order oligomers [6], the effect of ADA on the quaternary structure of A_{2A}R-A_{2A}R homomers was analyzed by BRET experiments. Cells were co-transfected with 0.15 µg of the cDNA encoding for A_{2A}R-Rluc and increasing amounts of the cDNA corresponding to A_{2A}R-YFP. 48 h post transfection cells were treated (20 min at 37°C) with medium or with 1 µg/ml of ADA in medium, and BRET was measured. In the absence of ADA the hyperbola obtained upon increasing the acceptor expression indicated a specific interaction between the two fusion proteins (Fig. 2). The BRET_{max} was 43 ± 3 mBU and the BRET₅₀ was 9 ± 2. The specificity of the A_{2A}Rs homomerization was confirmed by the unspecific (linear) BRET signal obtained in cells co-transfected with the cDNA corresponding to A_{2A}R-Rluc and increasing amounts of the cDNA corresponding to GABA_{B2}-YFP receptor (Fig. 2). Interestingly, in the presence of ADA, a significant (P < 0.01) increase in the BRET_{max} was observed (60 ± 2 mBU) without significant alterations in BRET₅₀ (9 ± 1). These results can be interpreted in two ways. In one, ADA led to conformational changes in A_{2A}Rs homomers that reduces the distance between Rluc and YFP fused to the C-terminal domain of the two A_{2A}R-containing fusion proteins. In the other, ADA increase the receptor homomerization by increasing the affinity between protomers. In this last case, a decrease in the BRET₅₀ values could be expected as there is binding between monomers to give homomers, BRET₅₀ might represent the affinity between protomers. Since the BRET₅₀ values were not changed in the presence of ADA we favor the first interpretation, that of ADA causing conformational changes.

ADA modulated the agonist and antagonist binding to A_{2A}Rs

The effect of ADA on ligand binding to A_{2A}Rs was first determined using A_{2A}Rs expressed in a more physiological context. For this purpose striatal membranes, which express a high amount of A_{2A}R, were selected. Isolated membranes were incubated with increasing concentrations of ADA and 17 nM of the radiolabelled A_{2A}R agonist ([³H]CGS 21680, see Experimental section). ADA enhanced in a dose-dependent manner the agonist binding to A_{2A}Rs (Fig. 3a) with an EC₅₀ value of 0.26 ± 0.03 ng/ml, which approximately corresponds to 6 pM. To test whether the effect of ADA was independent of its enzymatic activity, a preparation containing an irreversible-inhibited enzyme was used. ADA was inactivated using a preparation containing 100 µM Hg²⁺; non-bound Hg²⁺ was removed by gel filtration prior to the

assays (see Experimental section). Membrane suspensions were incubated with 17 nM [³H]CGS 21680 in the absence or in the presence of 1 µg/ml of active or Hg²⁺-inactivated ADA. Both, active or Hg²⁺-inactivated ADA enhanced to a similar extent agonist binding to striatal A_{2A}Rs (Fig. 3a insert), thus demonstrating that the effect was independent of the enzyme activity and suggesting that in our exhaustively washed membrane preparation there is not enough endogenous adenosine to interfere with the ligand binding to receptors. ADA also enhanced the A_{2A}R antagonist [³H]ZM 241385 binding to striatal membranes in a dose-dependent manner (Fig. 3b) with an EC₅₀ value of 0.13 ± 0.06 ng/ml, which is approximately equivalent to 3 pM ADA. Purified bovine serum albumin (1 to 10 nM) did neither modify agonist nor antagonist binding to striatal A_{2A}Rs, showing the ADA effect was specific (results not shown). All these results suggest that ADA is an allosteric modulator of A_{2A}Rs.

To further investigate the modulating effect of ADA on agonist and antagonist binding, the pharmacological parameters for ligand binding to A_{2A}Rs were calculated by means of saturation and competition experiments. To investigate the modulating effect of ADA on the A_{2A}R antagonist equilibrium dissociation constants, brain striatal membranes were incubated with increasing concentrations of [³H]ZM 241385 in the absence or in the presence of 1 µg/ml ADA, and saturation experiments were performed as indicated in Experimental section. Since A_{2A}Rs are expressed as dimers or higher order oligomers [6], radioligand saturation curves were analyzed by fitting the specific binding data to the mechanistic two-state dimer receptor model [32, 33], that considers a homodimer as the minimal structural unit of the receptor. In the absence or in the presence of ADA the saturation curves (Fig. 4a) were monophasic (D_c = 0) according to the non-cooperative behaviour of ZM 241385 binding to A_{2A}Rs [35]. The resulting equilibrium constants from fitting data to eq. 2 were 4.6 ± 0.8 nM and 1.9 ± 0.4 nM in the absence or in the presence of ADA respectively (mean ± S.E.M. of three different assays). This effect of ADA on antagonist affinity was also analyzed by competition binding experiments with 1.6 nM [³H]ZM 241385 and increasing concentrations of ZM 241385 in the absence or in the presence of 1 µg/ml ADA. In the absence or in the presence of ADA the competition curves (Fig. 4b) were also monophasic (D_c = 0). The resulting equilibrium constants from fitting data to eq. 5 were 5.1 ± 0.7 nM and 3.3 ± 0.8 nM in the absence or in the presence of ADA respectively (mean ± S.E.M. of three different assays), not significantly different from saturation parameters. Thus, ADA significantly (p<0.05) increased the affinity of A_{2A}Rs for the antagonist.

To determine the modulating effect of ADA on the A_{2A}R agonist CGS 21680 equilibrium dissociation constants, we only carried out competition binding experiments since saturation experiments with a low affinity ligand are not reliable. Radioligand binding was therefore determined in brain striatal membranes incubated with a constant amount of [³H]CGS 21680 (17 nM) and increasing concentrations of CGS 21680, in the absence or presence of 1 µg/ml ADA. As shown in Figure 5, competition curves of [³H]CGS 21680 vs CGS 21680 were monophasic (D_c = 0) according to the non-cooperative behavior expected for CGS 21680 binding [37]. The resulting equilibrium constant from fitting data to eq. 5 were 90 ± 20 nM and 41 ± 4 nM in the absence or in the presence of ADA respectively (mean ± S.E.M. of three different assays). Thus, ADA also significantly (p<0.05) increased the affinity of A_{2A}Rs for the agonist.

Signalling consequences of the ADA-A_{2A}R interaction

To investigate the functional consequences of the interaction of ADA with A_{2A}Rs, the A_{2A}R-mediated signal transduction was determined in cells expressing the receptors. Accordingly, CHO-A_{2A}R cells were treated for 5 min at 37° with increasing

amounts of the $A_{2A}R$ agonist, CGS 21680, in the absence or presence of 1 $\mu\text{g/ml}$ ADA, and ERK 1/2 phosphorylation was determined as indicated in the Experimental section. In the absence of ADA, CGS 21680 up to 200 nM dose-dependently increased ERK 1/2 phosphorylation followed by a decrease of signaling at high CGS 21680 concentrations (Fig. 6). The phenomenon in which previous or continued exposure of receptor to agonist results in a diminished functional response of the receptor upon subsequent or sustained agonist treatment has been defined as desensitization [38]. It has been described that A_{2A} receptors-mediated adenylate cyclase stimulation desensitizes rapidly in cultured cells (see [38] for review). Our results suggest that in A_{2A} receptors expressing CHO cells there is also a CGS 21680-promoted desensitization of ERK 1/2 phosphorylation. In the presence of ADA a significant increase in the CGS 21680-induced ERK 1/2 phosphorylation was observed resulting also in a bell-shaped concentration-response curve (Fig. 6). According to an ADA-induced increase in ligand affinity for $A_{2A}Rs$, ADA also increased the $A_{2A}Rs$ signalling, determined as ERK 1/2 phosphorylation. These results show that ADA not only increased ligand affinity for $A_{2A}Rs$, but also was able to modulate, in a positive manner, signal transduction. ADA may then be considered an enhancer of ligand binding and of $A_{2A}R$ -mediated signaling events.

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DISCUSSION

Cell surface ADA needs to be anchored to the plasma membrane by means of specific receptors. Here we describe that ADA may bind to A_{2A}Rs on the surface of living cells. By fluorescence or bioluminescence resonance energy transfer techniques (FRET and BRET) it has previously been demonstrated that A_{2A}Rs form homomers and that homomers but not monomers appear to be the functional species at the cell surface of transfected cells [6]. Thus, the quaternary structure of A_{2A}Rs is constituted by, at least, two protomers that form a dimer. Likely resulting from a decrease in the distance between the C-termini of the A_{2A}R protomers fused to Rluc and YFP, ADA binding led to modifications in the quaternary structure of A_{2A}R homomers that could be detected by BRET experiments. Using a similar set up Canals et al., [6] showed that A_{2A}R agonists are not able to modify the BRET signal. Therefore, the ability of BRET to detect ADA-triggered conformational changes within the A_{2A}R homomers suggests that ADA exerts a control of the function of A_{2A}R homomers by a strong modification of their quaternary structure. In fact, the ADA-induced structural changes in the A_{2A}R molecule correlated with marked affinity modifications in the binding of both agonist and antagonist. Irrespective of its enzymatic activity ADA was able to significantly decrease agonist and antagonist equilibrium dissociation constants. The ADA-induced increase in the ligand affinities indicates that ADA behaved as a positive modulator of A_{2A}Rs.

In addition to orthosteric sites, many GPCRs have been found to possess structurally distinct allosteric domains. One characteristic feature of the allosteric interaction is that the receptor is able to simultaneously bind an orthosteric and an allosteric ligand, introducing complexity into pharmacological responses by modifying the affinity or the signal imparted by the orthosteric ligand [39]. An allosteric effect results in a positive modulation if the modulator facilitates the interaction, or in a negative modulation if it inhibits the interaction of the ligand with the orthosteric binding site [39, 40]. According to these concepts, ADA is an allosteric ligand of A_{2A}Rs that positively modulates the agonist and antagonist binding to the orthosteric site of the receptor. Kreth et al., [41] have shown that an endogenous allosteric modulator leads to a reduced ligand affinity and to an impaired function of the adenosine A_{2A}R of human granulocytes in sepsis. Furthermore, some compounds have been synthesized and evaluated as positive enhancers of agonist and antagonist radioligands for the neuronal A_{2A}R [42, 43]. A_{2A}Rs are allosterically modulated by sodium ions binding to an allosteric site linked to Glu13 in TM1 and His278 in TM7, and by the potassium sparing diuretic, amiloride [43-45]. The ability of allosteric modulators to fine tune pharmacological responses has sparked interest in their potential applications in both clinical and basic science settings [40]. This interest is more relevant in the case of neurotransmitter receptor targets due to the fact that synaptic neurotransmission occurs in extremely complex circuits implicated in many neurological functions. Due to the A_{2A}Rs implication in many neurodegenerative diseases such as Parkinson, Huntington, obsessive-compulsive disorders and drug addiction [46], different approaches have been tested to find allosteric modulators, i.e., a structure-based ligand discovery methodology provided new routes for modulation of this neuronal key target [47-49]. Conceptually the allosteric interaction here described is different from the one exerted by small molecules since it comes from the interaction across the membrane with a protein that has an extracellular topology. By means of the interaction with an extracellular domain of A_{2A}Rs, ADA exerts a fine tune modulation of adenosine neuroregulation that may have important implications for the function of neuronal A_{2A}Rs, which are enriched in and play a key role in the brain striatum. The presence of ADA bound to the cell surface

of neurons has been demonstrated [22], reinforcing the concept that this allosteric effect of ADA is likely to occur in vivo. With this in mind one may hypothesize that ADA SCID patients with ADA mutations affecting the binding of ADA to A_{2A}R may manifest neurological alterations that are predicted to be different from those resulting from mutations not affecting the ADA-A_{2A}R interface. Probably, mutations affecting the interaction would be less deleterious for striatal function since it would attenuate overactivation of A_{2A}R exerted by the elevated adenosine levels. Irrespective of this, the results here described show that ADA, apart from reducing the adenosine concentration, binds to A_{2A}R behaving as an allosteric effector that markedly enhances agonist-induced signaling through MAPK pathway, increasing ERK 1/2 phosphorylation. Thus, the physiological role of the ADA-adenosine receptors interaction is to make those receptors more functional.

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FIGURE LEGENDS

Figure 1. Expression of ADA on the cell surface of wild type and A_{2A}R expressing CHO cells. Non-permeabilized wild type CHO cells (b and d) or CHO-A_{2A}R cell clone (a and c) were labeled with FITC-anti-A_{2A}R antibody (a and b) or with FITC-anti-ADA antibody (c and d). Cells were processed for confocal microscopy analysis as described under Experimental section.

Figure 2. Effect of ADA on A_{2A}R homomerization detected by BRET experiments. BRET saturation experiments were performed as described in the Experimental section using cells transfected with 0.15 µg of cDNA corresponding to A_{2A}R-*Rluc* and increasing amounts of cDNA corresponding to A_{2A}R-YFP (0.8 to 3 µg cDNA) (squares and circles) or to GABA_{B2}-YFP receptor (0.3 to 3 µg cDNA) as a negative control (triangles). After 48 h transfection, cells were treated for 20 min with medium (circles and triangles) or with 1 µg/ml ADA (squares) before BRET determination. Both fluorescence and luminescence for each sample were measured before every experiment to confirm similar donor expressions (approximately 120,000 bioluminescence units) while monitoring the increase in acceptor expression (10,000 to 50,000 fluorescence units). The relative amount of BRET is given as the ratio between the fluorescence of the acceptor (YFP) and the luciferase activity of the donor (*Rluc*). BRET data are expressed as means ± S.E.M. of three to four different experiments grouped as a function of the amount of BRET acceptor.

Figure 3. Effect of ADA on A_{2A}R agonist and antagonist binding to brain striatal membranes. Binding of 17 nM [³H]CGS 21680 (a) or 1.6 nM [³H]ZM 241385 (b) to striatal membranes (0.3 mg of protein/ml) was performed as described in the Experimental section, in the presence of increasing concentrations of ADA. Data points on the y-axis correspond to the binding in the absence of ADA. Insert in (a): 17 nM [³H]CGS 21680 binding in the absence (white bar) or in the presence of 1 µg/ml of active (grey bar) or Hg²⁺-inactivated (black bar) ADA was performed as described above. Data are mean ± S.E.M. of triplicates. Significant differences respect to the samples in absence of ADA were calculated by unpaired Student's t test (* p<0.05).

Figure 4. Effect of ADA on A_{2A}R antagonist affinity constants. (a) Saturation binding experiments of increasing concentrations of de radiolabelled antagonist [³H]ZM 241385 (0.1 to 27 nM) or (b) competition experiments of the antagonist [³H]ZM 241385 (1.6 nM) binding *versus* increasing concentrations of ZM 241385, in the absence (black circles) or in the presence (white circles) of 1 µg/ml ADA. Data are mean ± S.E.M. from a representative experiment (n=3) performed in triplicate.

Figure 5. Effect of ADA on A_{2A}R agonist affinity constants. Competition experiments of the agonist [³H]CGS 21680 (17 nM) binding versus increasing concentrations of CGS 21680, in the absence (black circles) or in the presence (white circles) of 1 µg/ml ADA. Data are mean ± S.E.M. from a representative experiment (n=3) performed in triplicate.

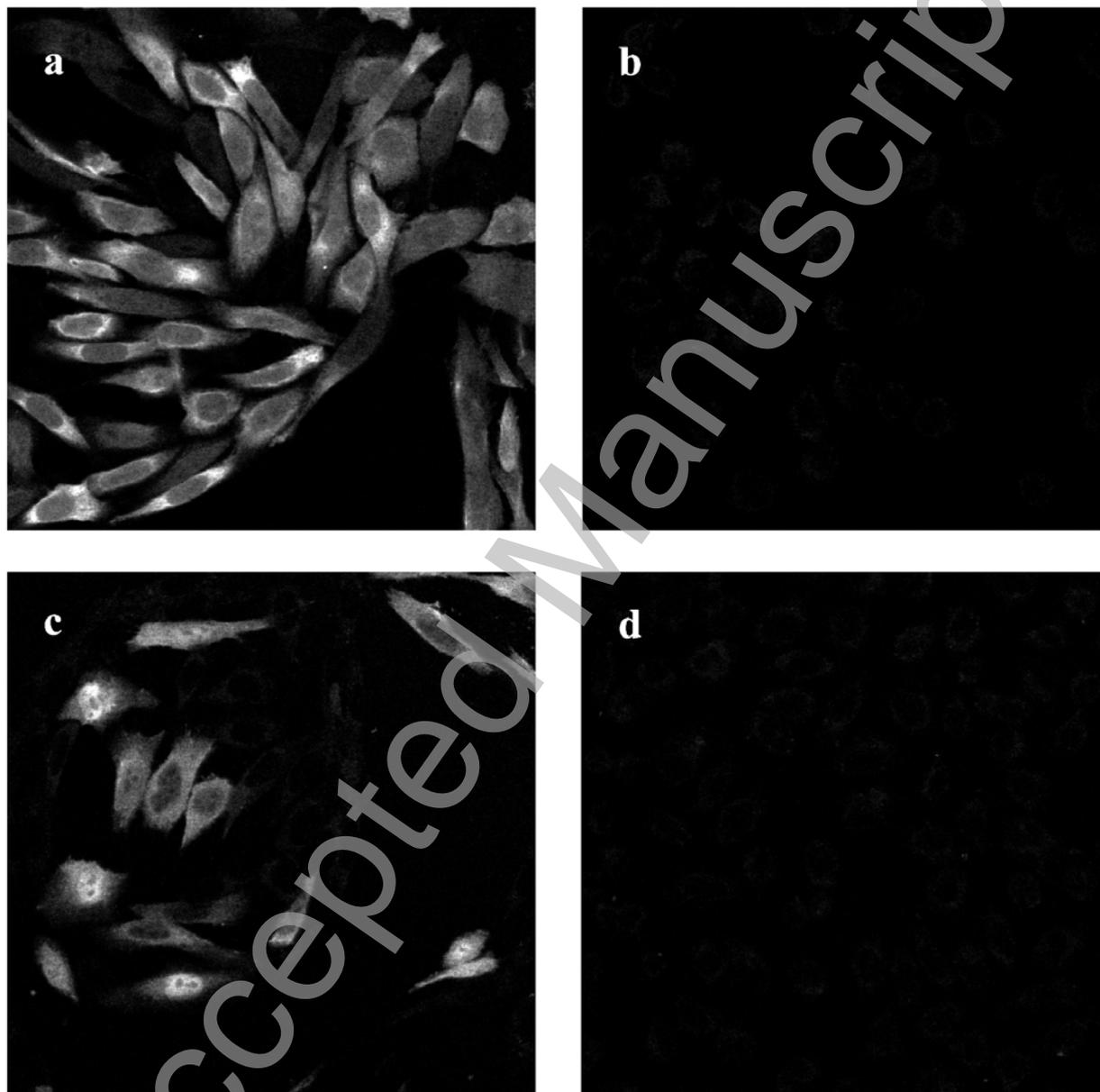
Figure 6. Effect of ADA on A_{2A}R-mediated ERK 1/2 phosphorylation. A_{2A}Rs expressing CHO cells were stimulated with increasing concentrations of the A_{2A}R agonist CGS 21680 in the presence (black columns) or in the absence (white columns) of 1 µg/ml ADA. In (a) a representative western-blot is shown. In (b) values are mean ± S.E.M. of three independent

experiments. Significant differences respect to the samples in absence of ADA were calculated by unpaired Student's t test (* $p < 0.05$ and ** $p < 0.01$).

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Figure 1



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Figure 2

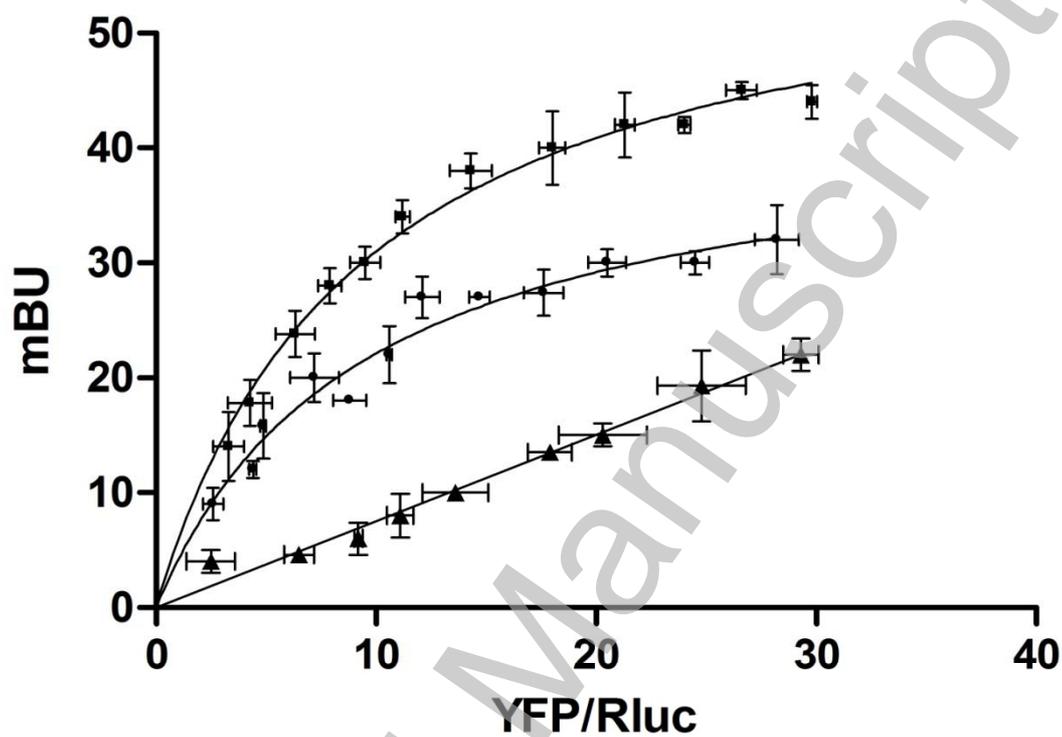
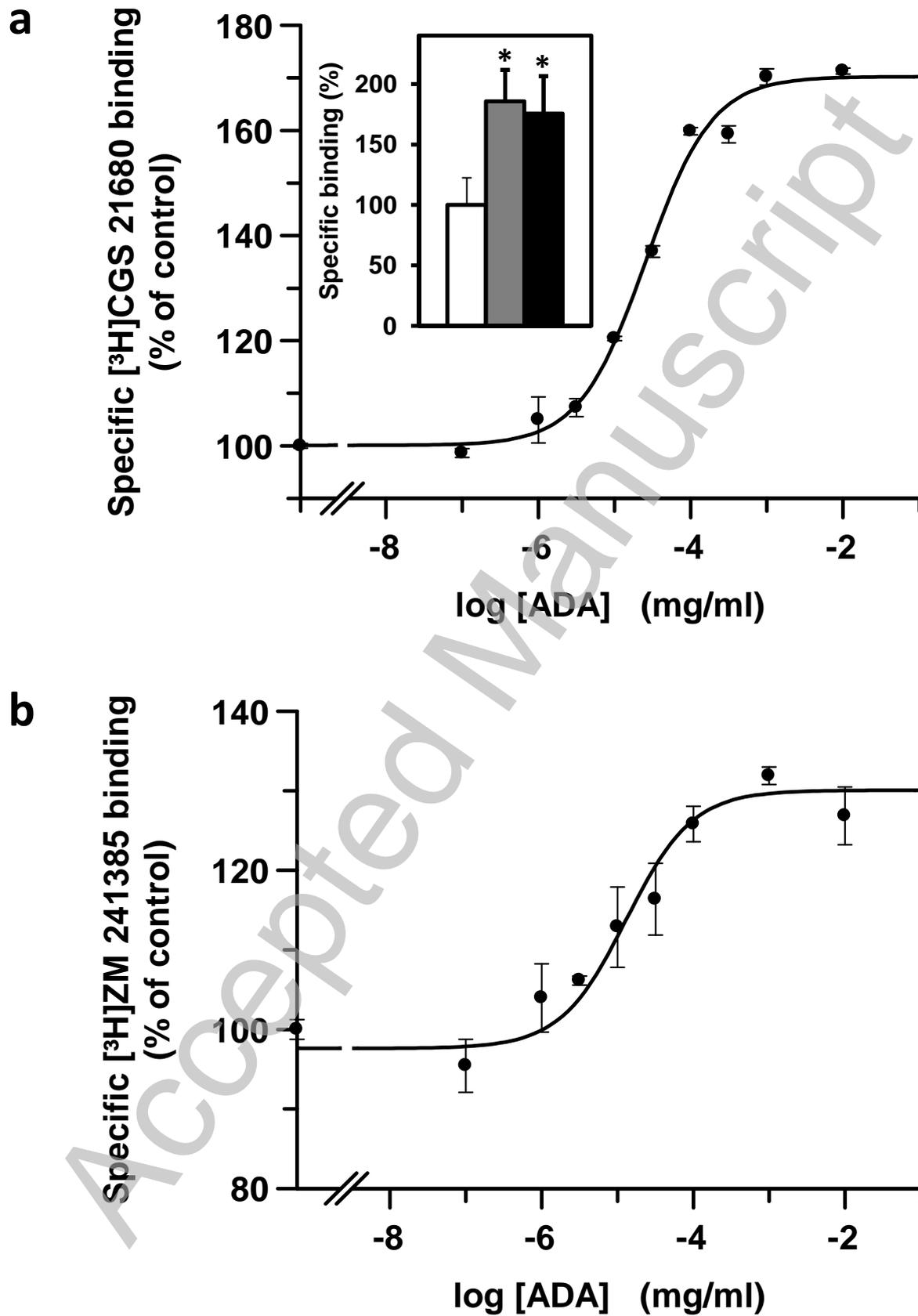
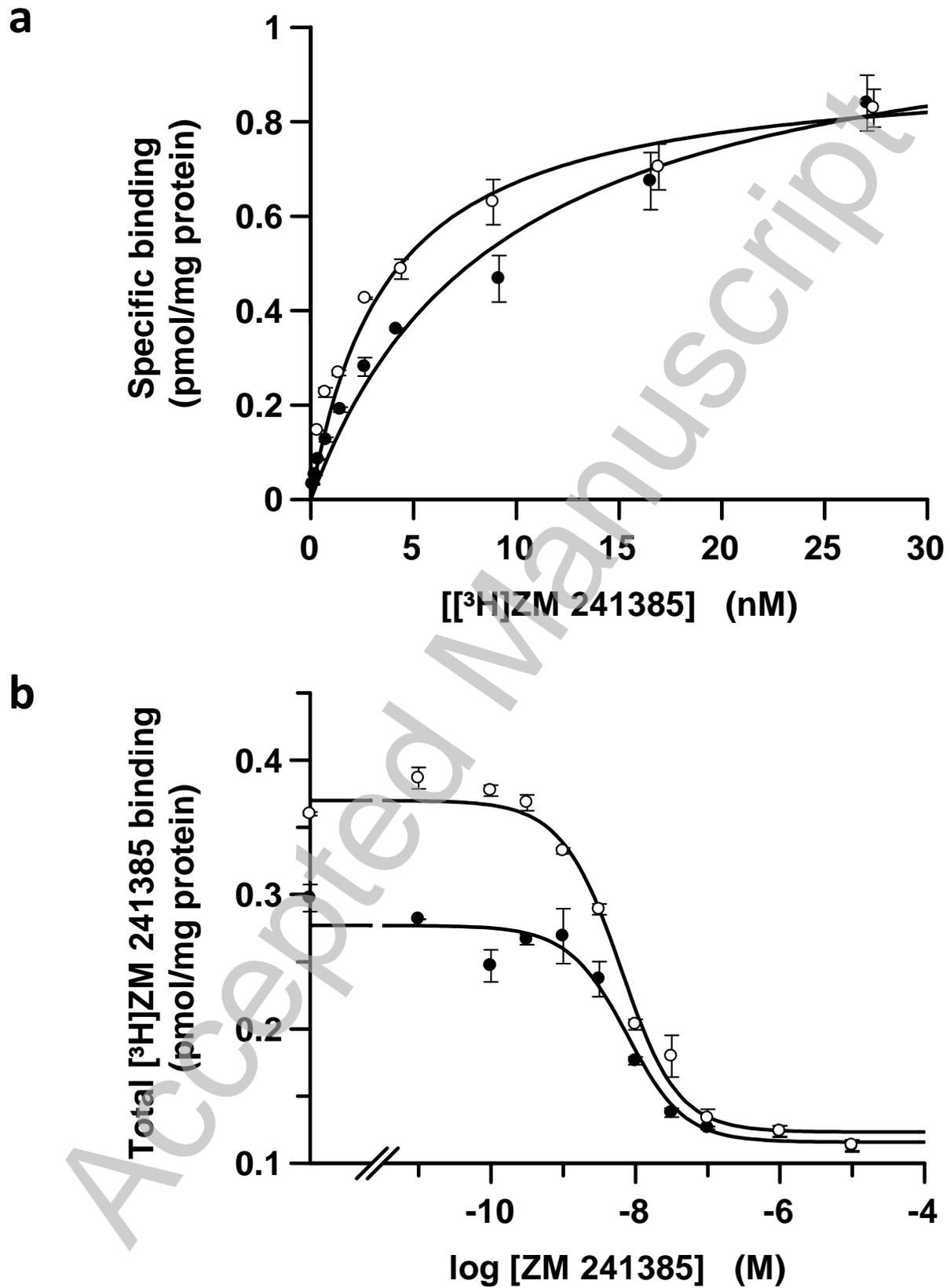


Figure 3



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Figure 4



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Figure 5

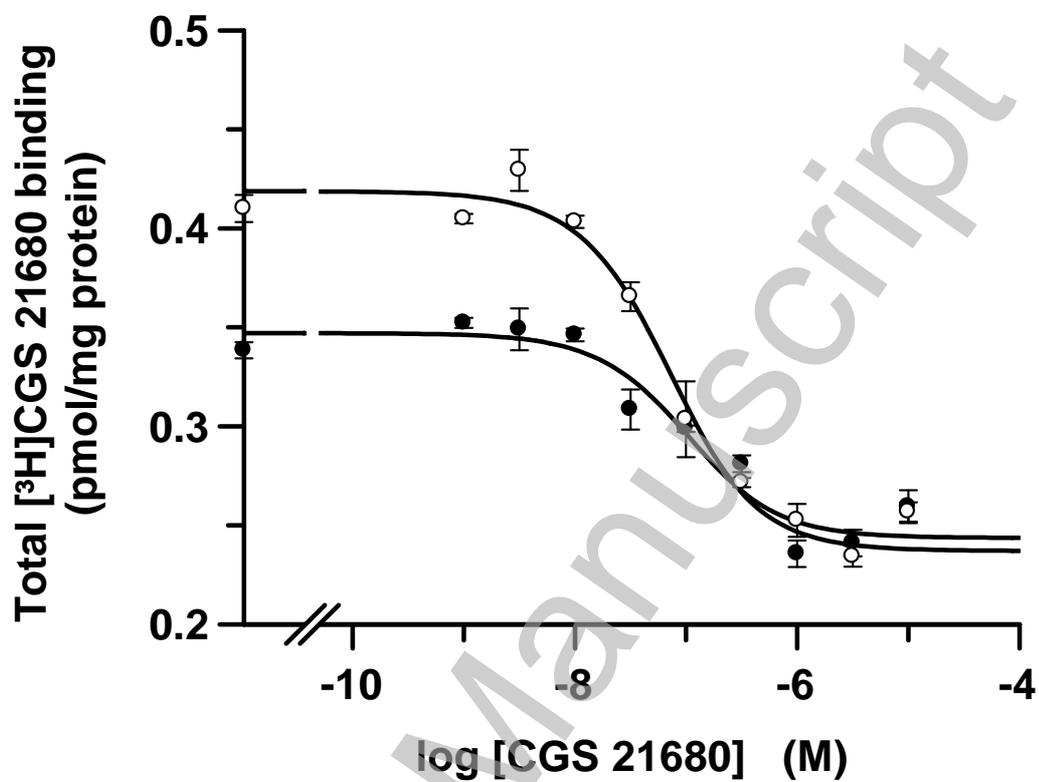
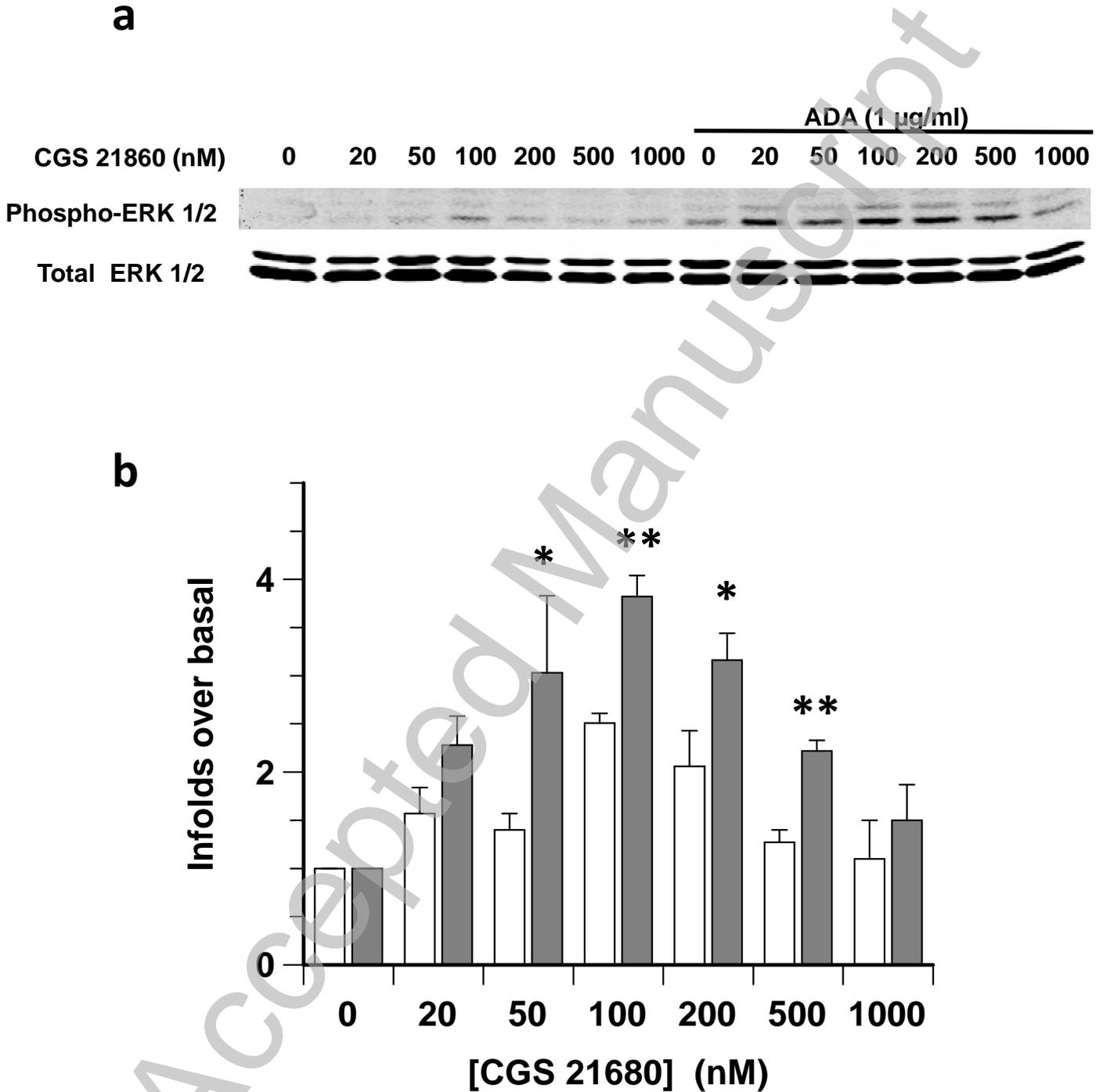


Figure 6



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