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Abstract

Resveratrol (RSV) is a natural polyphenolic antioxidant with a proven protective role in several human diseases involving oxidative stress, although the molecular mechanism underlying this effect remains unclear. The present work tried to elucidate the molecular mechanism of RSV's role on signal transduction modulation. Our biochemical analysis, including radioligand binding, real time PCR, western blotting and adenylyl cyclase activity, and computational studies provide insights into the RSV binding pathway, kinetics and the most favored binding pose involving adenosine receptors, mainly A2A subtype. In this study, we show that RSV target adenosine receptors (AdoRs), affecting gene expression, receptor levels, and the downstream adenylyl cyclase (AC)/PKA pathway. Our data demonstrate that RSV activates AdoRs. Moreover, RSV activate A2A receptors by directly binding to the classical orthosteric binding site. Intriguingly, RSV-induced receptor activation can stimulate or inhibit AC activity depending on concentration and exposure time. Such subtle and multifaceted regulation of the AdoRs/AC/PKA pathway might contribute to the protective role of RSV. Our findings suggest that RSV molecular action is mediated, at least in part, by activation of adenosine receptors and create the opportunity to interrogate the therapeutic use of RSV in pathological conditions involving AdoRs, such as Alzheimer.

Keywords	Resveratrol; adenosine receptors; GPCR modulation; C6 glioma cells.
Taxonomy	Natural Product Biochemistry, Receptor-Ligand Dynamics, Protein Function Relationship

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Departamento de Química Inorgánica, Orgánica y Bioquímica

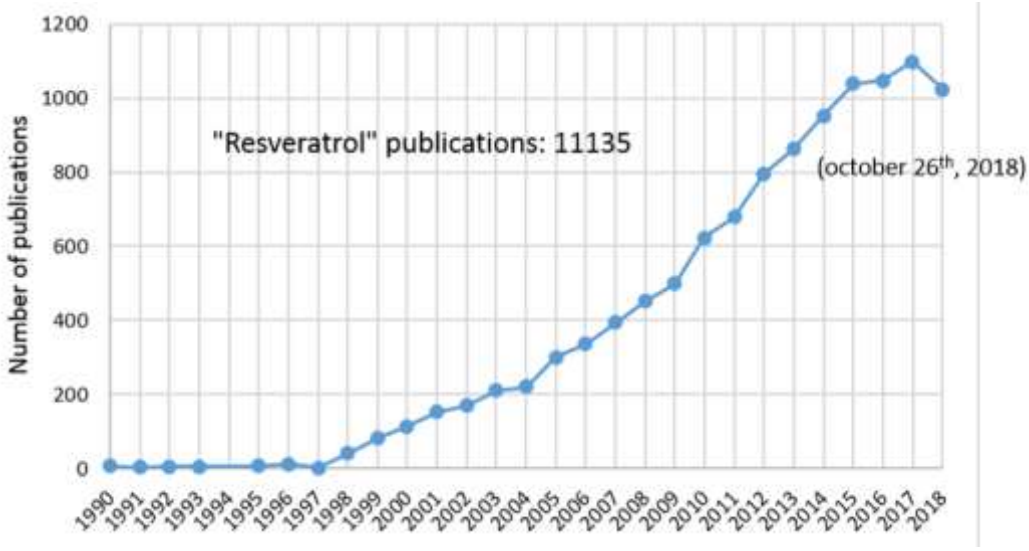
Kelvin J. A. Davies,
Editor-in-Chief
Free Radical Biology & Medicine

October 26th, 2018.

Dear Editor in Chief,

Please find enclosed the manuscript entitled "*THE ANTIOXIDANT RESVERATROL ACTS AS A NON-SELECTIVE ADENOSINE RECEPTOR AGONIST*", which we would like to be considered for publication in *Free Radical Biology & Medicine* journal.

The therapeutic potential of resveratrol (RSV) has been discussed extensively due to its antioxidant and positive effects on diseases such as cancer, cardiovascular diseases, ischemia, chronic metabolic, inflammatory and neurodegeneration disorders, among others. Accordingly, there is increasing interest in this molecule as shows the number of publications (11135) obtained from PubMed using "resveratrol" as the search term.



However, the precise molecular mechanism underlying RSV's action has not yet been established.

In the present work, we combine biochemical, pharmacological and computational approaches to provide the first evidence that RSV acts as a non-selective ligand at adenosine receptors. We find that RSV modulates the adenosine receptor-mediated signaling pathway in C6 glioma cells, which endogenously express all four subtypes of adenosine receptor (A_1, A_{2A}, A_{2B} and A_3). Our data strongly suggest that the observed effects of RSV, including gene expression and abundance of the receptors, as well as the activity of the corresponding AC pathway, are produced by direct binding of RSV to adenosine receptors. Finally, we conclude that the modulatory effect of RSV treatment is mainly mediated by binding to the A_{2A} receptor, where RSV seems to acts as an agonist.

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Since adenosine receptors have been involved in many disorders, such as neurodegenerative diseases, where adenosine has been showed to be neuroprotective, we propose that adenosine receptors mediate the potent and beneficial effects of this polyphenol. We cannot exclude additional molecular targets of RSV, but the fact that RSV binds to adenosine receptors, which are localized at the plasma membrane, indicates that this is the first place where cells responds to RSV, perhaps followed by other intracellular targets.

For these reasons, we believe that our results will be interesting for readers of Free Radical Biology & Medicine.

We looking forward to hearing your response.

Yours sincerely,

Dr. José Luis Albasanz.

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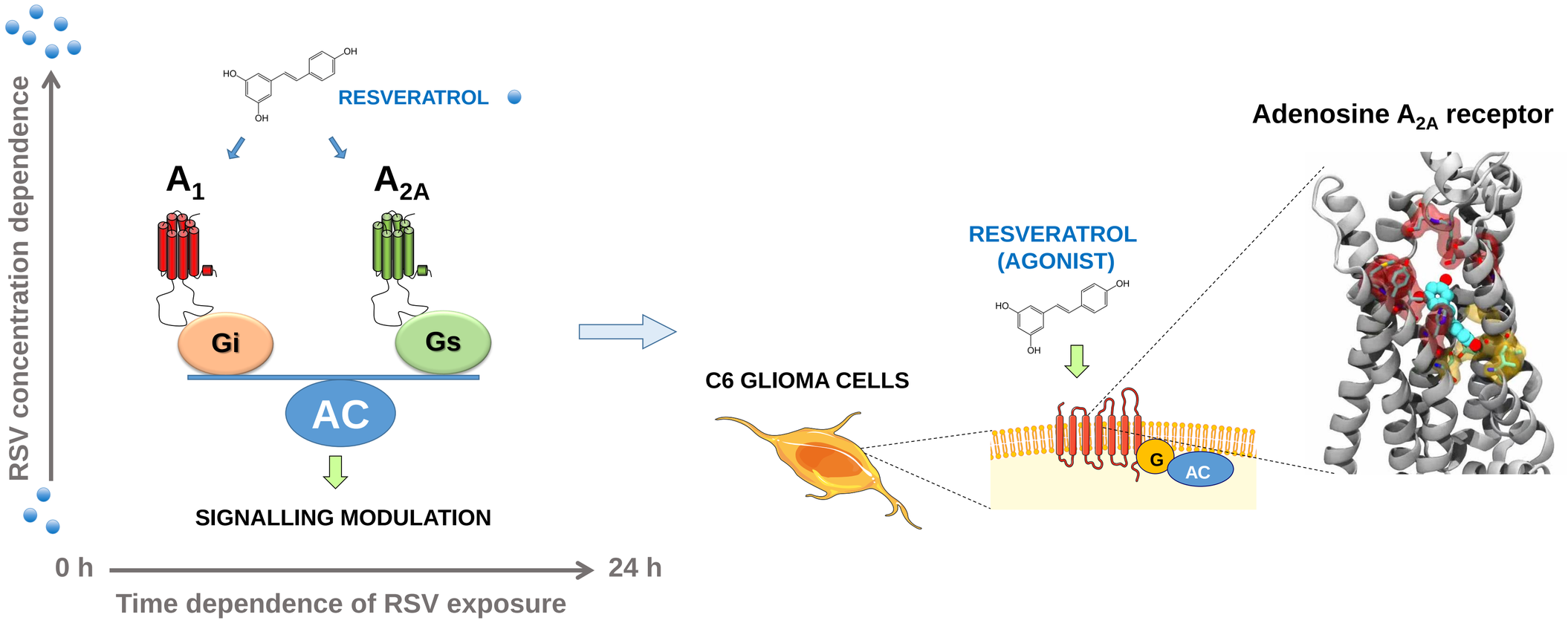
THE ANTIOXIDANT RESVERATROL ACTS AS A NON-SELECTIVE ADENOSINE RECEPTOR AGONIST

- First evidence of resveratrol as a non-selective ligand at adenosine receptors.
- Resveratrol modulates adenosine receptors-mediated signaling in C6 glioma cells.
- Resveratrol directly binds to agonist binding site of the A_{2A} receptor.
- Plasma membrane could be the first place where cells responds to resveratrol.


RESVERATROL
A1
A2A
Gi
Gs
AC
SIGNALLING MODULATION
RSV concentration dependence
0 h
24 h
Time dependence of RSV exposure
C6 GLIOMA CELLS
RESVERATROL
(AGONIST)
G
AC
Adenosine A2A receptor

THE ANTIOXIDANT RESVERATROL ACTS AS A NON-SELECTIVE ADENOSINE RECEPTOR AGONIST

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Running tittle: resveratrol activates adenosine receptors

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Keywords: Resveratrol; adenosine receptors; GPCR modulation; C6 glioma cells.

ABSTRACT

Resveratrol (RSV) is a natural polyphenolic antioxidant with a proven protective role in several human diseases involving oxidative stress, although the molecular mechanism underlying this effect remains unclear. The present work tried to elucidate the molecular mechanism of RSV's role on signal transduction modulation. Our biochemical analysis, including radioligand binding, real time PCR, western blotting and adenylyl cyclase activity, and computational studies provide insights into the RSV binding pathway, kinetics and the most favored binding pose involving adenosine receptors, mainly A_{2A} subtype. In this study, we show that RSV target adenosine receptors (AdoRs), affecting gene expression, receptor levels, and the downstream adenylyl cyclase (AC)/PKA pathway. Our data demonstrate that RSV activates AdoRs. Moreover, RSV activate A_{2A} receptors by directly binding to the classical orthosteric binding site. Intriguingly, RSV-induced receptor activation can stimulate or inhibit AC activity depending on concentration and exposure time. Such subtle and multifaceted regulation of the AdoRs/AC/PKA pathway might contribute to the protective role of RSV. Our findings suggest that RSV molecular action is mediated, at least in part, by activation of adenosine receptors and create the opportunity to interrogate the therapeutic use of RSV in pathological conditions involving AdoRs, such as Alzheimer.

INTRODUCTION

Resveratrol (RSV) is a natural polyphenolic phytoalexin with a stilbene structure (trans-3', 4', 5'-trihydroxystilbene) that is mostly found in grape skin, seeds and red wines. It is produced by plants, where it is protective against stressful environmental conditions. In the early 1990s, RSV was highlighted as a promising molecule by numerous *in vivo* and *in vitro* studies that reported its biological properties and its ability to attenuate diseases such as cancer, cardiovascular diseases, ischemia, chronic metabolic, inflammatory and neurodegeneration disorders, mainly focusing on its antioxidant properties [1-3]. In fact, a search for RSV's actions in the PubChem database (https://pubchem.ncbi.nlm.nih.gov, CID445154) results in 3893 entries in 3051 bioassays where RSV's effects on various targets has been assessed and compiled. From these bioassays, 764 are classified as *Active*, i.e. that RSV produced an effect in the analyzed target/assay (e.g. cell viability assays, inhibition of enzyme activity, etc.). Proteins are the target of 1298 bioassays, involving 803 proteins analyzed from which 124 have been reported to have modified expression or function, and could therefore be suggested as protein targets for RSV. In addition, several proteins have been crystallized in complex with RSV (see Supplemental Table 1) revealing this molecule's diverse modes of action. However, the precise molecular interaction has not yet been established.

Many studies suggest that RSV acts by modulating the activity of several enzymes and other proteins, although its protective role is not yet well understood [4]. RSV reduces cellular hydrogen peroxide, and increases both glutathione content and MnSOD expression, which enhances antioxidant defenses [5]. RSV has been reported to significantly reduce cell death induced by $A\beta_{25-35}$ and Nitric Oxide (NO) in primary cultured hippocampal cells. RSV has also been found to attenuate neuroinflammation by reducing the release of pro-inflammatory cytokines in microglial culture, suggesting a neuroprotective role of this polyphenol [6]. Many reports propose the cAMP/PKA/AMPK signaling pathway as a target for RSV, as this pathway is clearly increased after RSV treatment; again, the underlying molecular mechanism remains unclear [7]. Additionally, since RSV has a similar structure to hormones such as estrogens, it

can bind estrogen receptors and produce phytoestrogen-like activity without changes in activation of the cAMP/AMPK/PKA signaling pathway [8].

Despite this evidence on the therapeutic potential of RSV *in vivo* [3, 9], there is still disagreement between *in vivo* and *in vitro* results, which may be partly due its low bioavailability and rapid metabolism by the liver [1]. This controversy has led some authors to propose that RSV-derived metabolites may also exhibit some activity in cells, or even that these metabolites account for some of the effects previously described for RSV. This is the case for RSV glucuronides (RSV 3-O-D-glucuronide, RSV 4-O-D-glucuronide, and RSV 3-O-sulfate), which inhibit cell growth by G1 arrest and cyclin D1 depletion in colon cancer cells. Interestingly, this inhibitory effect has been suggested to involve the adenosine A_3 receptor [10]. Furthermore, the cardioprotective effect of RSV in rat heart may be due to its ability to stimulate adenosine release [11]. Oral RSV intake in humans, mainly in the form of red wine, leads to increased adenosine plasma levels, which could contribute to the beneficial effect of moderate red wine consumption in reducing cardiovascular risk [12], as observed in epidemiological studies in populations whose daily diet includes wine [13].

Adenosine is a purine nucleoside that is widespread in the body, where it is involved in physiological processes ranging from neuromodulation to immune system regulation, vascular function and control of energy balance [14]. Adenosine is the major natural ligand for adenosine receptors (AdoRs), which are G-protein-coupled receptors (GPCR) classified as A_1, A_{2A}, A_{2B} and A_3 receptors. A_1 and A_3 inhibit adenylyl cyclase (AC) through Gi/o proteins, while A_{2A} and A_{2B} stimulate AC through Gs proteins [15]. AdoRs have been linked to human diseases [16]. In fact, the adenosinergic system is clearly altered in several neurodegenerative diseases, including Alzheimer's disease (AD) in which the A_1 and A_{2A} receptors are significantly increased in the frontal cortex of *post mortem* human brains [17]. RSV has been related to neuroprotection [18-20] and seems to stimulate adenosine release [11, 12]. Moreover, we recently reported that diet supplemented with RSV modulates the adenosine-

mediated signaling pathways in SAMP8 mice, a model of AD, suggesting a protective role by re-sensitizing A_1 and desensitizing A_{2A} mediated signaling in RSV-treated mice. Therefore, as RSV modulates and reverses the age-related effect on adenosine-mediated signaling in SAMP8 mice [21], the aim of this study was to identify the molecular mechanism underlying RSV's action on the adenosinergic system. We assessed whether RSV modulates AdoRs and their inhibitory and/or stimulatory signaling pathways in C6 glioma cells that endogenously express AdoRs [22]. Our results show that RSV binds and modulates AdoRs function, suggesting that the neuroprotective role of RSV could be mediated by this direct binding to AdoRs.

MATERIALS AND METHODS

Chemicals

Cyclopentyl-1,3-dipropylxanthine-8-[dipropyl-2,3–^{3}H(N)] ([^{3}H]DPCPX 120 Ci/mmol) (ref. NET974) and [^{3}H]cAMP (ref.NET275) were from NEN. ([2–^{3}H](4-(2-[7-amino- 2-(2-fury1)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino] ethyl)phenol) ([^{3}H]ZM241385 27.4 Ci/mmol) (ref.ART-884) was from American Radiolabeled Chemicals. N-[9-Chloro-2-(2-furanyl)[1,2,4]-triazolo[1,5-c]quinazolin-5-yl]benzene acetamide (MRS 1220) (ref.1217) and 2-[p-(2-carboxyethyl) phenylamino]-5'-N-ethylcarboxamido adenosine (CGS 21680) (ref.1063) were from Tocris. *Trans*-Resveratrol (RSV) (ref.R5010) N^6-cyclopentyladenosine (CPA) (ref.C-8031), N^6-cyclohexyladenosine (CHA) (ref.C-9901) and theophylline (ref.T-1633) were from Sigma Aldrich. Calf intestine adenosine deaminase (ADA) (ref.10102121001) from Roche.

Cell culture

Rat C6 glioma cells were grown in Dulbecco´s modified Eagle´s medium (DMEM) supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 1% non-essential aminoacids

and antibiotics in a humidified atmosphere of 95% air, 5% CO_2 at 37°C, as previously described [22]. Cells were treated with RSV at a final concentration of 100 μM for 24 h. Rat C6 glioma cells were obtained from American Type Culture Collection (ref-CCL-107).

Quantification of adenosine A_1 and A_{2A} receptors by radioligand binding assays in intact cells

Radioligand binding assays were performed in intact cells as described previously [22], with some modifications. $3x10^4$ cells/well were seeded in 24-well plates. To remove endogenous adenosine, cells were pre-incubated with 5 U/ml ADA at 25°C for 30 min. Then, binding assays were performed using [^{3}H]DPCPX or [^{3}H]ZM241385 as selective radioligands for A_1 or A_{2A} receptors, respectively, at saturation concentration (20 nM), as previously described [22]. CPA (4 mM), a selective A_1 receptor agonist, or theophylline (9 mM) were used to obtain non-specific binding to A_1 or A_{2A} receptors, respectively. After incubation at 25°C for 2 h, cells were washed with ice-cold serum-free DMEM to remove the free radioligand, and were then disrupted with 0.2% SDS. Well contents were then transferred to vials, and scintillation liquid mixture was added in order to count radioactivity in a Microbeta Trilux counter (Perkin Elmer). At least two wells from each plate were reserved for protein concentration measurements.

Competition binding experiments

Intact cells were used to construct competition binding curves. 10^5 cells/well were seeded in 24-well plates. After 24 hours, cells were pre-incubated with 5 U/ml ADA at 25°C for 30 min to remove endogenous adenosine. Competition assays were performed using RSV at different concentrations (0.1-500 μM) as a displacing ligand and 20 nM [^{3}H]DPCPX or 20 nM [^{3}H]ZM241385 as selective radioligands for the A_1 and A_{2A} receptors, respectively. After incubation (2h at 25°C), cells were washed with serum free DMEM to remove the free radioligand and disrupted with 0.2% SDS. Well contents were transferred to vials and scintillation liquid mixture was added to count radioactivity.

Total RNA isolation and preparation of cDNA

Total RNA was extracted from cells using an ABI 6100 Nucleic Acid PrepStation and chemicals according to the manufacturer's protocol (Applied Biosystems, Madrid, Spain) as previously described [22]. The A_{260}/A_{280} ratio (RNA purity) was in the range 1.9-2.1. RNA concentrations were determined from the A_{260}. One microgram of total RNA was reverse transcribed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, ref.4368813) according to manufacturer's protocol.

Quantitative Reverse-Transcription Polymerase Chain Reaction

Relative gene expression was assessed, as previously described [22], by quantitative real time PCR analysis performed in an AB Prism 7500 Fast system, using TaqMan probes and primers from Applied Biosystems: A_1 (Rn00567668_m1), A_{2A} (Rn00583935_m1), A_{2B} (Rn00567697_m1), A_3 (Rn00563680_m1), Adenylyl cyclase-I (Rn02115682_s1) and β-actin (Rn00578826_m1). β-actin was used as an endogenous control for normalization.

Preparation of cell homogenate

Control and RSV treated cells were homogenized in a buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.4) containing a mixture of protease inhibitors (100 μM PMSF and 100 μg/mL Bacitracin) in Dounce homogenizer (10xA, 10xB). After homogenization and determination of protein concentrations, samples were stored at -80°C until assays were performed.

C6 glioma cells plasma membrane isolation

Plasma membrane isolation was performed as previously described [22]. Control and RSV-treated cells homogenates were centrifuged at 12000g for 30 minutes. Pellets were re-suspended in the homogenization buffer, the protein concentration was determined using the Lowry Method, and the samples were then stored at -80°C.

Western blotting assays

To perform Western blotting assays, 30 µg of protein was mixed with loading buffer (0.125 M Tris-HCl pH 6.8, 20% glycerol, 10% β-mercaptoethanol, 4% SDS and 0.002% bromophenol blue), and heated at 95°C for 5 minutes. SDS polyacrylamide gel electrophoresis (10% SDS-PAGE) was carried out using a mini-protean system (Bio-Rad, Madrid, Spain) with molecular weight standards (Bio-Rad). Proteins were transferred to nitrocellulose membranes, which were washed with TTBS containing 10 mM Tris-HCl (pH 7.4), 140 mM NaCl and 0.1% Tween-20, blocked with TTBS containing 5% skimmed milk, and then incubated with the primary antibodies at 4°C overnight. The antibodies used were anti-PKA (1:500 dilution) (Assay Biotech, ref.B0548), anti-A_{2B} (1:200 dilution, Santa Cruz Biotechnology, ref.SC-7507), anti-A_3 (1:1000 dilution, Millipore, ref.AB1590P), anti-Adenylyl cyclase I (1:1000 dilution, Abcam, ref.ab8226) and anti-β-actin (1:2000 dilution, Abcam, ref.ab38331). After rinsing, the membranes were incubated with the corresponding secondary antibodies (1:5000, Bio-Rad, Madrid, Spain). The immunoreaction was visualized using the enhanced chemiluminescence (ECL Prime) detection Kit (GE Healthcare, Madrid, Spain, ref.RPN2236), and the specific bands were quantified in a G:Box densitometer and normalized with β-actin using GeneTools software (Syngene, UK).

Determination of adenylyl cyclase activity in intact cells

Adenylyl cyclase (AC) activity was determined in intact cells as previously described [22]. Briefly, cells were washed with serum-free DMEM and pre-incubated with 100 µM Ro 20-1724 and 2 U/ml ADA for 15 min at 37°C to remove endogenous adenosine. AC activity was then induced with various ligands for 15 min at 37°C. The reaction was stopped by adding 0.1 M HCl in absolute ethanol, and cells were then transferred to microtubes. Ethanol was evaporated in a SpeedVac concentrator and the pellet was re-suspended in assay buffer (50 mM Tris-HCl, 4 mM EDTA, pH 7.5) to determine cAMP accumulation, using protein kinase A as cAMP-binding protein and [^{3}H]cAMP as radioligand. Standard samples were prepared in the same buffer in a range of 0-16 pmol. The reaction was disrupted by rapid filtration in a Filtermate harvester (PerkinElmer) with filters previously incubated with (poly)ethylenimine at

3% for 30 minutes. Scintillation liquid mixture was added to filters in order to count radioactivity. At least two wells from each condition (control, RSV treated) were reserved for measuring protein concentrations.

Molecular Dynamics simulations

We used VMD1.9 [23] to set up the initial structure, visualize and analyze all Molecular Dynamics simulations. The crystal structure of the adenosine A_{2A} receptor with PDB code 4EIY was retrieved from the PDB database. Residue 1 (MET) was modeled into the structure and residues from the apocytochrome b(562)RIL plus all heteroatoms and ligands were omitted. Crystallization water molecules within 5 Å of the receptor were retained. One RSV molecule was then placed randomly at the extracellular side 10 Å away from the receptor and the CHARMM-GUI builder [24] was used to model and embed the receptor into a pure 1-palmitoyl-2-oleyl-sn-glycero-3-phosphocholine (POPC) bilayer of approximately 90 x 90 $Å^2$. HIS264 was protonated, whereas all titratable residues of the receptor were left in the dominant protonation state at pH 7.4. The system was then solvated, neutralized, and the ionic strength adjusted. All simulations were performed using the CHARMM36 [25] and CHARMM27 [26] force fields to represent POPC and proteins, respectively. RSV parameters were generated with the CHARMM General Force Field [27] with partial charges generated from AM1-BCC calculations [28]. Excess water molecules was adjusted using VMD, and the system was then geometry-optimized, and equilibrated with harmonic positional restraints to lipids and C_α atoms of the protein that were sequentially released in a gradual manner. Metadynamics simulations were performed according to a recently published protocol [29]. We used a combination of the well-tempered variant (WT) [30, 31] of metadynamics and funnel metadynamics [32]. A metadynamics history-dependent bias was applied along the component of the z-distance between the relatively immobile Cα of Trp6.48 deep in the ligand-binding region and the center of mass of the ligand's heavy atoms. This distance was used as the single collective variable. The funnel restraint was then applied to the relative position on the xy-plane (restrained to 8 Å radius when fully unbound and allowing 13 Å radius if around

the vestibule or deeper) to ensure better sampling for the relevant region of the free energy. Otherwise, the ligand can move extensively in the extracellular solvent without affecting the free energy. Gaussian hills with an initial height of 0.48 kcal mol^{-1} were applied every 1 ps. The hill width was chosen to be 1 Å. The Gaussian functions were rescaled in the WT scheme using a bias factor of 20. Representative structures were extracted throughout the simulation of initial docking for each 2 Å window, and used as starting coordinates for the multiple walker technique [33]. This ensured faster convergence of the free-energy surface and enhanced the parallelization. Free energies were calculated using the PLUMED plug-in [34], as described in our protocol [29].

Statistical analysis

Statistical parameters and statistical significance are reported in the Figures and Figure Legends. Differences between mean values were considered statistically significant when $p < 0.05$ by two-tailed Student's t test. In figures, asterisks denote statistical significance as calculated by Student's t test (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$). Statistical analysis was performed in GraphPad PRISM 6 program (GraphPad Software, San Diego, CA, USA).

RESULTS

Resveratrol modulates gene expression and protein levels of adenosine receptors

To establish whether RSV affects AdoRs gene expression, we treated C6 cells with 100 μM RSV for 24 h and isolated total RNA. We then performed real-time PCR assays to study the expression of all four AdoRs genes (ADORA1, ADORA2A, ADORA2B, ADORA3) and isoform I of the adenylyl cyclase (AC) gene, and found a high and significant increase in A_{2A} and A_{2B} gene expression after RSV treatment. In contrast, we observed a significant decrease in A_3 gene expression and no significant differences in A_1 and AC-I gene expression between control and RSV-treated cells (Figure 1).

Next, we used radioligand binding in intact cells and Western blotting assays of the plasma membrane fraction to analyze whether the observed change in gene expression caused by RSV was also observed at the protein level. We observed a significant increase in A_1 and A_3 receptors in RSV-treated cells (Figure 2, panel A and E, respectively), but no significant differences in A_{2A} or A_{2B} receptors (Figure 2, panel B and D, respectively).

Resveratrol overstimulates basal AC activity and desensitizes the A_{2A} receptor-mediated pathway

We next performed AC activity assays to analyze whether the main transduction pathway of AdoRs, the cAMP/PKA/AMPK pathway, is modulated by RSV treatment. We found that both the basal activity of AC (Figure 3, panel A) and the corresponding protein level (panel B) were significantly increased after exposure to RSV as compared with control cells, indicating that the enzymatic activity of AC is significantly modified. In contrast, A_1-mediated inhibition of forskolin-stimulated AC activity by CHA, a selective A_1 agonist, was not significantly altered by RSV treatment, as compared with control cells (Figure 3, panel C). However, A_{2A}-mediated stimulation of AC activity by CGS 21680, a selective A_{2A} agonist, decreased significantly (Figure 3, panel D). In addition, Western blot assay showed a significant increase in protein kinase A (PKA) levels after exposure to RSV for 24 hours (Figure 4).

Resveratrol binds to A_1 and A_{2A} receptors

To study how RSV modulates AdoRs and to determine whether RSV acts directly or indirectly on these receptors, we next used radioligand binding assays to construct competition curves in intact control cells, with RSV as the displacing ligand for [^{3}H]DPCPX- and [^{3}H]ZM241385-specific binding to A_1 and A_{2A} receptors, respectively. RSV could displace both selective radioligands in a concentration-dependent manner, which highlights A_1 (Figure 5, panel A) and A_{2A} (Figure 5, panel B) receptors as a possible direct target for RSV. IC_{50} values of RSV were of approximately the same order for A_1 and A_{2A} receptors (83.4 ± 13.4 μM for [^{3}H]DPCPX, and

47.3 ± 12.8 μM for [^{3}H]ZM241385) with no significant differences between them. As IC_{50} values can depend on assay conditions, we calculated the Ki value using the Cheng-Prusoff equation, with Kd values for A_1 and A_{2A} receptor in C6 cells as previously published by our group [22]. Accordingly, we obtained a Ki of 39.1 ± 6.3 μM for A_1, and a Ki of 21.3 ± 5.8 μM for A_{2A} receptors. These values were not significantly different, suggesting that RSV has similar affinity for both AdoRs. Enhanced-sampling molecular dynamics further corroborated that the most energetically preferred binding site for RSV is the orthosteric binding site of the A_{2A} receptors (see below).

Resveratrol acts as a non-selective adenosine receptor agonist

As RSV was able to displace binding of selective radioligands, such as [^{3}H]DPCPX and [^{3}H]ZM241385, from A_1 and A_{2A} receptors, respectively, we assessed whether RSV can act as an AdoRs agonist or antagonist. To this end, we determined AC activity in controls cells treated with RSV concentrations ranging from 1-150 μM. As shown in Figure 6, RSV induced a significant increase in AC activity at higher concentrations (50-150 μM), but not at lower concentrations (1-10 μM). This AC activity was completely abolished when A_{2A} receptors were selectively blocked by the antagonist ZM241385 (Figure 6), suggesting that RSV stimulates AC activity by binding to the A_{2A}-receptor. In contrast, when we analyze the effect of RSV on forskolin-stimulated AC activity, we only observe significant inhibition at lower concentrations, but not at higher concentrations (Figure 7, panel A). Moreover, RSV's ability to inhibit AC was reverted in the presence of DPCPX, a selective A_1 antagonist, or MRS 1220, a selective A_3 antagonist (Figure 7, panel B), suggesting that the effect of RSV (i.e. AC inhibition) is also mediated by binding to A_1 and A_3. These results suggest that RSV has an agonistic effect on AdoRs.

The modulatory effect of RSV treatment on AC activity is mediated by A_{2A} receptors

RSV's ability to modulate AC activity by binding to stimulatory (A_{2A}) and inhibitory (A_1, A_3) receptors would depend on its concentration and its affinity for AdoRs. Thus, in control cells

RSV activates both A_1 and A_{2A} receptors, resulting in a net AC stimulation, which becomes more evident with increasing RSV concentration (Figure 6).

Since RSV treatment modulated AdoRs (protein levels and gene expression), AC (basal activity and protein) and PKA (protein), we assessed the AdoRs-mediated response to RSV in cells treated with 100 μM RSV for 24 hours, i.e. the effect of RSV on AC activity in RSV-pre-treated cells. We observed that RSV had an inhibitory effect (Figure 8, panel A), in contrast to its stimulatory effect in control cells (Figure 6). We found a similar result when we compared the effect of RSV on AC activity in both control and RSV-pre-treated cells to that obtained with 10 μM CGS 21680, a selective A_{2A} agonist. Thus, the percentage of stimulation of AC activity by 10 μM CGS 21680 or 100 μM RSV in control cells was of the same order. Moreover, the effect of RSV on AC activity in cells after RSV treatment was again similar to and of the same order as that observed in the presence of 10 μM CGS 21680 (Figure 8, panel B). Since RSV mimics the effects of the selective A_{2A} agonist, these results suggest that RSV acts mainly through A_{2A} receptors.

To reinforce these results, we assessed whether the selective blockade of A_{2A} could avoid the increase in basal AC activity observed with RSV treatment. To do this, we analyzed the effect of selective A_{2A} antagonism by measuring basal AC activity after treatment with RSV or RSV + ZM241385, both at 100 μM for 24 h (Figure 8, panel C). We found no significant differences in basal AC activity when cells were treated only with the selective A_{2A} antagonist ZM241385 compared to the control. However, we observed a significant reduction in basal AC activity when RSV-treated cells were co-treated with ZM241385 (Figure 8, panel C), confirming that the modulatory effect of RSV is mainly mediated by A_{2A} receptors.

The most energetically favored binding site for RSV is the orthosteric binding site

To interrogate RSV binding to the A_{2A} receptor at a molecular level, we conducted metadynamics simulations, using the z projection of the distance between RSV and the protein

to define the reaction coordinate (see Methods and Figure 9, panel A). During the initial simulations, RSV samples the extracellular site of the protein and binds the vestibule region before accessing its orthosteric binding pocket (see Supplemental Movie S1). The free energy profile reconstructed from converged simulations reveals three energy minima that correspond to binding sites in the receptor's vestibule (3), and the orthosteric binding site (1 and 2) (see Supplemental Movie S2). Interestingly, the energetically most favored binding site energy minima 1 broadly overlaps with that of the previously crystallized ZM241385 (ZMA) ligand (Figure 9, panel B). This observation is in line with our result showing that RSV can displace ZM241385 from the binding pocket (Figure 5, panel B). Both RSV and ZM241385 are sandwiched between hydrophobic residues F168 (extracellular loop 2) and L6.51 (highlighted in bold, Figure 9, panel B). The smaller molecular RSV is further stabilized by polar interactions with N5.42. In addition, a water-network towards the pocket opening links RSV to E196 in extracellular loop 2. Our free energy binding profile suggests that RSV binds to the A_{2A} receptor rather weakly, with an estimated Ki value of 40 μM. Importantly, this weak binding value agrees with experimentally determined affinities for the A_1 (Ki=39.1 ± 6.3 μM) and A_{2A} receptors (Ki=21.3 ± 5.8 μM), indicating the potential physiological relevance of the observed binding pose.

DISCUSSION

There have been many published reports on the biological effects of RSV since it was first proposed as the main molecule responsible for the protective effects of red wine consumption against coronary heart disease. RSV has been shown to be a cardioprotective [13, 35], chemopreventive and chemotherapeutic [36], and neuroprotective compound that can cross the blood brain barrier [19, 20]. It has even been suggested as a therapeutic agent for AD [3, 6, 37, 38].

While there have been many reports on the effects of RSV, little is known about its molecular mechanisms of action. Previous studies have suggested that RSV is involved in activating the cAMP/PKA/AMPK signaling pathway, but the mechanism remains unclear [4, 7, 39, 40]. RSV activates adenylyl cyclase (AC) in MCF-7 cells in a time- and concentration-dependent manner [8], and binds to other receptors such as estrogens receptors [41, 42]. Note that estrogens receptor activation by estradiol has been reported to have a neuroprotective role [43]. However, it has been also suggested that activation of the cAMP/AMPK/PKA pathway by RSV is independent of estrogen receptors [8]. While RSV acts on different intracellular cascades, such as PDE, cAMP, AMPK or sirtuin [2, 39], its primary molecular target has not yet been described.

Importantly, our study provides strong evidence that AdoRs are a primary target for RSV. We show that RSV can directly bind AdoRs, where it acts as a non-selective ligand with affinity of approximately the same order as for the A_1 (Ki=39.1 ± 6.3 μM) and A_{2A} (Ki=21.3 ± 5.8 μM) receptors. This binding has functional consequences as in control cells, RSV increases AC activity in a concentration-dependent manner. This effect is similar to that observed with CGS 21680, a selective A_{2A} agonist, and is reverted by the presence of ZM241385, a selective A_{2A} antagonist. This indicates that the observed increase in AC activity is mainly mediated by adenosine A_{2A} receptors. RSV also inhibits forskolin-stimulated AC activity similarly to CHA, a selective A_1 agonist. Interestingly, this inhibition is only detectable at low RSV concentrations (1-10 μM). Our data suggest that higher concentrations simultaneously stimulate the A_{2A} receptor, which is linked to enhanced AC activity, thus masking the A_1-induced AC inhibition. This can be explained by the similar affinity values of RSV (IC_{50}=83 μM) and the selective A_1 receptor agonists R-PIA (IC_{50}=1.6 μM) and CHA (IC_{50}=4.2 μM). In contrast, the affinity of RSV for the A_{2A} receptor (IC_{50}=47 μM) is significantly lower than that of the selective A_{2A} agonist CGS 21680 (IC_{50}=4.2 nM), as previously described in C6 cells [22]. In line with this, it has recently been reported that AdoRs form heterotetramers (two A_1 and two A_{2A}) with a quaternary structure with two G-proteins (Gi and Gs) [44] that enhance the diversity of

mechanisms through which extracellular stimuli are transferred to G-protein coupling. This heterotetramer would act as an adenosine concentration-sensing device [45]. At low concentrations, Gi is engaged *via* A_1 activation without engaging Gs signaling. At higher concentrations Gs is engaged *via* A_{2A} activation, and this engagement blocks Gi-mediated signaling. Taken together, our data indicate that RSV has a concentration-dependent agonistic effect: (i) A_1 receptor agonist at lower concentration, and (ii) dual A_1 and A_{2A} receptor agonist at higher concentration (Supplemental Scheme 1). A_{2A} activation has previously been suggested, but not fully demonstrated, in rats with steroid-induced ocular hypertension, in which topical *trans*-RSV lowers intraocular pressure, but only at low doses [46]. However, we recently reported the modulation of both A_1 and A_{2A} receptors in RSV-treated SAMP8 mice [21].

In our paper, we used a very sensitive technique, radioligand binding, to show that RSV can selectively bind the A_1 and A_{2A} receptors, and this is corroborated by computational studies. Molecular dynamics simulations show that RSV accesses the A_{2A} receptor *via* the extracellular side, first binding to the vestibule of the A_{2A} receptor and then entering the orthosteric site. The most preferred binding pose largely overlaps with that of the A_{2A} selective antagonist ZMA, which explains its ability to compete with ZMA for binding. The binding kinetics indicate that RSV is a weak binder, which is consistent with experimentally obtained values, and supports the physiological relevance of the detected binding pose. Our study further shows that binding to A_1 and A_{2A} receptors affects the AC/PKA pathway in this glial cell model.

In RSV's role as an AdoRs agonist, these receptors become regulated after prolonged RSV exposure (Supplemental Scheme 1). Thus, RSV exerts a different effect on AC activity when cells are previously treated with the same polyphenol. After treatment, RSV behaves as an inhibitory agent on the basal enzymatic activity and losses its stimulatory action on AC activity detected in control cells. This RSV effect on AC activity was exactly the same as observed after treatment with CGS 21680 for 24 hours, and was diminished after RSV treatment in the

presence of ZM241385, again suggesting that RSV mainly exerts its effect through A_{2A} receptors. The opposite effect of RSV observed in control and RSV-pre-treated cells could be due to an increased abundance of A_1 receptors following RSV treatment, while the corresponding levels of A_{2A} receptors remain unchanged. It might also be due to A_{2A} receptors interacting with other receptors to form oligomers, dimers and heterotetramers in a complex with two different G-proteins [44, 47, 48]. This could change how extracellular signals are transferred to a specific signaling outcome. Accordingly, the cross-talk between A_1 and A_{2A} receptors in the brain and heart [49, 50] is well known; they even seem to form heteromers between themselves [47]. Moreover, no changes were found either in gene expression or in the activity of the corresponding A_1-mediated signaling pathway, suggesting that the increased abundance of A_1 receptors could be due to cross-talk mechanisms between A_1 and A_{2A} receptors [50]. On the other hand, the increased abundance of A_1 receptors was not paralleled by increased inhibition of AC activity by the selective A_1 agonist CHA, which could be due to altered coupling to Gi proteins. We observed a significant increase in expression of the ADORA2A gene after RSV treatment, which could be a compensatory mechanism, as CGS 21680 was unable to stimulate AC activity, or even inhibited this activity in RSV-treated cells. Accordingly, Wu and colleagues [51] used 2 μM CGS 21680 to induce A_{2A}-activation of human BeWo cells and observed increased expression of the ADORA2A gene *via* cAMP response element (CREB) signaling, which is consistent with previous reports showing that RSV modulates CREB-mediated gene transcription [52, 53].

Our results suggest that RSV treatment substantially alters the transfer of extracellular stimuli to intracellular signaling pathways. One possible explanation is that RSV switches the preference for A_{2A} receptor coupling from stimulatory to inhibitory G proteins (i.e. a Gs/Gi protein switch). Previously, Cunha and colleagues suggested that the A_{2A} receptor can also bind an inhibitory G-protein in the limbic cortex [54]. Moreover, similar Gs/Gi-protein switching has been reported for β-adrenoceptors in relation to immune system regulation [55] through phosphorylation of these receptors by PKA [56]. Changes in G-protein specificity can result

from the formation of oligomers containing A_{2A} and other receptors, as described for A_{2A}-CB_1 receptor heteromers in striatal cells [57]. Adenosine A_1-A_{2A} heterotetramers with two G-proteins, Gi and Gs, enhance the diversity of how extracellular stimuli can be transferred to G-protein coupling [44]. Modifying these heteromers could be important for the interplay of Gs- and Gi-mediated signaling, and may explain our results regarding A_{2A}-mediated signaling after RSV treatment. Another possible explanation for the observed inhibition of AC activity after 24 h treatment with RSV is that RSV switches from a Gs agonist into a Gs inverse agonist. In fact, caffeine, that directly binds to AdoRs and is widely considered as a non-selective antagonist, is able to act as an inverse agonist by reducing the constitutive activity of the A_{2A} receptor in some pathological conditions such as experimental parkinsonism [58].

Regardless of the regulatory mechanism, it is remarkable that RSV directly binds and modulates these receptors. Many reports have identified AdoRs, mainly A_{2A}, as new therapeutic targets, not only for neurodegenerative diseases [59], but also for cancer, ischemia, cardiovascular and autoimmune disorders, among others [60-62], although there is ongoing controversy as to how to approach these new therapies.

Altered levels of A_1 and A_{2A} receptors in patients with neurodegenerative diseases such as Alzheimer's [17] has led some authors to propose pharmacological blockade of AdoRs as therapy. Pharmacological blockade or genetic elimination of A_{2A} receptors could benefit patients with AD [63], by preventing memory loss, improving cognition [64], and also by attenuating A_{2A}-mediated neuroinflammation [65]. In agreement, several studies show that caffeine intake is associated with lower risk of AD [38]. Concerning cardiovascular disorders, A_{2A} activation seems to have an anti-atherosclerosis role by inhibiting the formation of foam cells [66]. Interestingly, the atheroprotective effects of RSV on cholesterol efflux in cells of the arterial wall was eliminated by pharmacological blockade of A_{2A} receptors with ZM-241385 [67]. Cancer and autoimmune disorders benefit from RSV protective effects [36, 68] which could be related to AdoRs. Thus, whereas A_{2A} activation inhibits the immune response, and

could thereby attenuate chronic inflammation and autoimmune disorders [69], A_{2A} blockade enhances antitumor activity by preventing immunosuppression of T-cells and Natural Killer cells [70].

As adenosine levels and its protective role may change depending on physiological or pathophysiological conditions [71], modulating adenosine levels could play a key role in situations where the pharmacological blockade of receptors have side effects [72]. There is clear evidence of altered purine metabolism in AD [73, 74]. In fact, A_1 and A_{2A} receptors are modulated in C6 cells exposed at hypoxic conditions through a mechanism involving endogenous adenosine [75]. Interestingly, RSV has also been reported to increase plasma levels of adenosine in human subjects [11, 12]. Moreover, RSV has been proposed as a possible antiepileptic, and its anticonvulsant activity seems to be mediated by an adenosinergic mechanism [18].

Therefore, as AdoRs have been involved in many disorders, such as neurodegenerative diseases, in which adenosine has been showed to be neuroprotective, we propose that AdoRs mediate the potent and beneficial effects of RSV. We cannot exclude additional molecular targets of RSV, of which estrogen receptor is perhaps one of the most likely. However, the fact that RSV binds to AdoRs, which are localized at the plasma membrane, highlights these receptors as the first place where cells responds to RSV, perhaps followed by other intracellular targets, such as estrogen receptor, as mentioned above.

In this paper, we combined biochemical, pharmacological and computational approaches to provide the first evidence of RSV as non-selective ligand at AdoRs. We find that RSV can modulate the AdoR-mediated signaling pathway in C6 glioma cells. Our data strongly suggest that the observed modulation, including gene expression, protein level and the activity of the corresponding AC pathway, is produced by direct binding of RSV to AdoRs. Finally, we conclude that the modulatory effect of RSV treatment is mainly mediated by binding to the A_{2A}

receptor, where RSV seems to acts as an agonist. Therefore, we suggest that the protective effect of RSV is mediated by AdoRs. We emphasize the potential of these receptors as targets for new therapies to deal with the pathologies in which they are involved.

CONCLUSIONS

The potent and beneficial effects of RSV have been related to its antioxidant properties and action on several molecular targets through mechanisms not yet fully defined. Our results demonstrate that RSV binds to AdoRs, which are localized at the plasma membrane, and highlights these receptors as the first place where cells responds to RSV, perhaps followed by other intracellular targets. Therefore, we propose that targeting AdoRs with RSV could be considered for future therapeutic strategies where these receptors have been involved, such as neurodegenerative diseases.

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FIGURE LEGENDS

Figure 1. Adenosine receptors and adenylyl cyclase-I mRNA expression after 24 h of 100 μM RSV treatment. Expression of adenosine A_1, A_{2A}, A_{2B} and A_3 receptors and AC-I mRNA, in control and RSV treated cells, was determined by real-time PCR using subtype-specific primers and TaqMan probes. Data are means ± SEM of 3-6 independent experiments each using different cDNA preparations. *p<0.05, **p<0.01, ***p<0.001 significantly different from control value according to Student *t* test.

Figure 2. Adenosine receptor abundance is modulated after 100 μM RSV treatment for 24 h. Specific binding in control and RSV treated cells was determined in intact cells at saturation concentration (20 nM) of [^{3}H]DPCPX for A_1 (A) and [^{3}H]ZM241385 for A_{2A} (B) receptors. Data are means ± SEM of 3-4 independent experiments in triplicate each using different preparations of intact cells. A_{2B} (D) and A_3 receptors (E) were detected by Western blotting assay in the membrane fraction (C). Data are means ± SEM of 3 independent experiments. β-actin was used as gel loading control. *p<0.05 significantly different from control value according to Student *t* test.

Figure 3. RSV treatment modulates Adenylyl Cyclase activity. (A) Basal AC activity represented as pmol/mg protein · min was determined after RSV treatment. (B) AC protein level was quantified by Western blotting assay in the membrane fraction. Data are means ± SEM of 3 independent experiments. (C) The A_1-mediated inhibition of AC activity was determined by incubating with 5 μM Forskolin + 100 μM CHA (a selective A_1 agonist) as compared with the stimulation of 5 μM Forskolin alone. (D) The A_{2A}-mediated stimulation of AC activity was determined by incubating with CGS 21680 (selective A_{2A} agonist) as compared with basal activity. All data in C and D are means ± SEM of 3-5 experiments performed in triplicate using different preparations. *p<0.05 and ***p<0.001 significantly different from

control values, and ##p<0.01 and ###p<0.001 significantly different from the corresponding basal AC activity, all according to Student *t* test.

Figure 4. RSV treatment increases protein kinase A (PKA) level. Level of PKA was detected by Western-blotting assay in homogenate preparations of control and RSV-treated cells. Data are means ± SEM of 3 independent experiments performed with different homogenate preparations. β-actin was used as gel loading control. *p<0.05 significantly different from control value according to Student *t* test.

Figure 5. RSV displaces radioligand binding to adenosine receptors in intact control cells. Competition binding assays were performed in control cells at saturation concentration (20 nM) of [^{3}H]DPCPX for A_1 (A) and [^{3}H]ZM241385 for A_{2A} (B) receptors in the absence (total binding) or the presence of increasing concentrations of RSV (0.1 to 500 μM). Data are means ± SEM of 3-4 independent experiments performed in triplicate, each using different cell preparations. IC_{50} values, determined by non-linear regression analysis, were 83.4 ± 13.4 μM for [^{3}H]DPCPX and 47.3 ± 12.8 μM for [^{3}H]ZM241385. These values were not significantly different according to Student *t* test.

Figure 6. Dose-response curve of RSV on adenylyl cyclase (AC) activity in control cells. AC activity was determined in intact control cells in the absence (basal) and the presence of different concentrations of RSV (10-150 μM). RSV-mediated AC stimulation was also determined in the presence of 100 μM ZM241385 (selective A_{2A} antagonist). Data are means ± SEM of 4-8 independent experiments performed in triplicate with different preparations. *p<0.05 and **p<0.01 significantly different from the corresponding value in the absence of ZM241385 according to Student *t* test.

Figure 7. Selective blockade of A_1 and A_3 receptors decreases RSV-mediated inhibition of adenylyl cyclase (AC) activity in control cells. (A) Effect of different concentrations of RSV (10-150 μM) on the inhibition of forskolin-stimulated AC activity. (B) Effect of the A_1 antagonist DPCPX and the A_3 antagonist MRS 1220 on RSV-mediated AC inhibition. Data are means ± SEM of 3-9 independent experiments performed in triplicate using different preparations. **p<0.01 and ***p<0.001 significantly different according to Student *t* test.

Figure 8. Effect of RSV and CGS 21680 on AC activity in control and RSV treated cells. (A) Effect of different concentrations of RSV (1-150 μM) on AC activity in cells pre-treated for 24 h with 100 μM RSV. (B) AC activity in the presence of 10 μM CGS 21680 or 100 μM RSV in control and RSV pre-treated cells. (C) Basal AC activity in cells treated with 100 μM RSV, 100 μM ZM241385 and 100 μM RSV + 100 μM ZM241385 for 24 h. Data are means ± SEM of 3-4 independent experiments performed in triplicate using different preparations. *p<0.05 and ***p<0.001 significantly different from basal value (A) or the indicated value (B, C), according to Student *t* test.

Figure 9. RSV binds to the A_{2A} receptor binding site. (A) Left, depiction of collective variable used in the simulations. Right, free energy profile of RSV binding along the reaction coordinate showing three energy minima (1 to 3) that correspond to three binding sites, one at the vestibule and two at the orthosteric binding site of the receptor. (B) Comparison of a representative RSV binding pose from energy minima 1 with A_{2A} selective antagonist ZM241385 from the PDB:3EML crystal structure. As shown in the left panel, both RSV (orange) and ZM241385 (green) adopt a similar binding pose with a common interacting network (middle and right panels).

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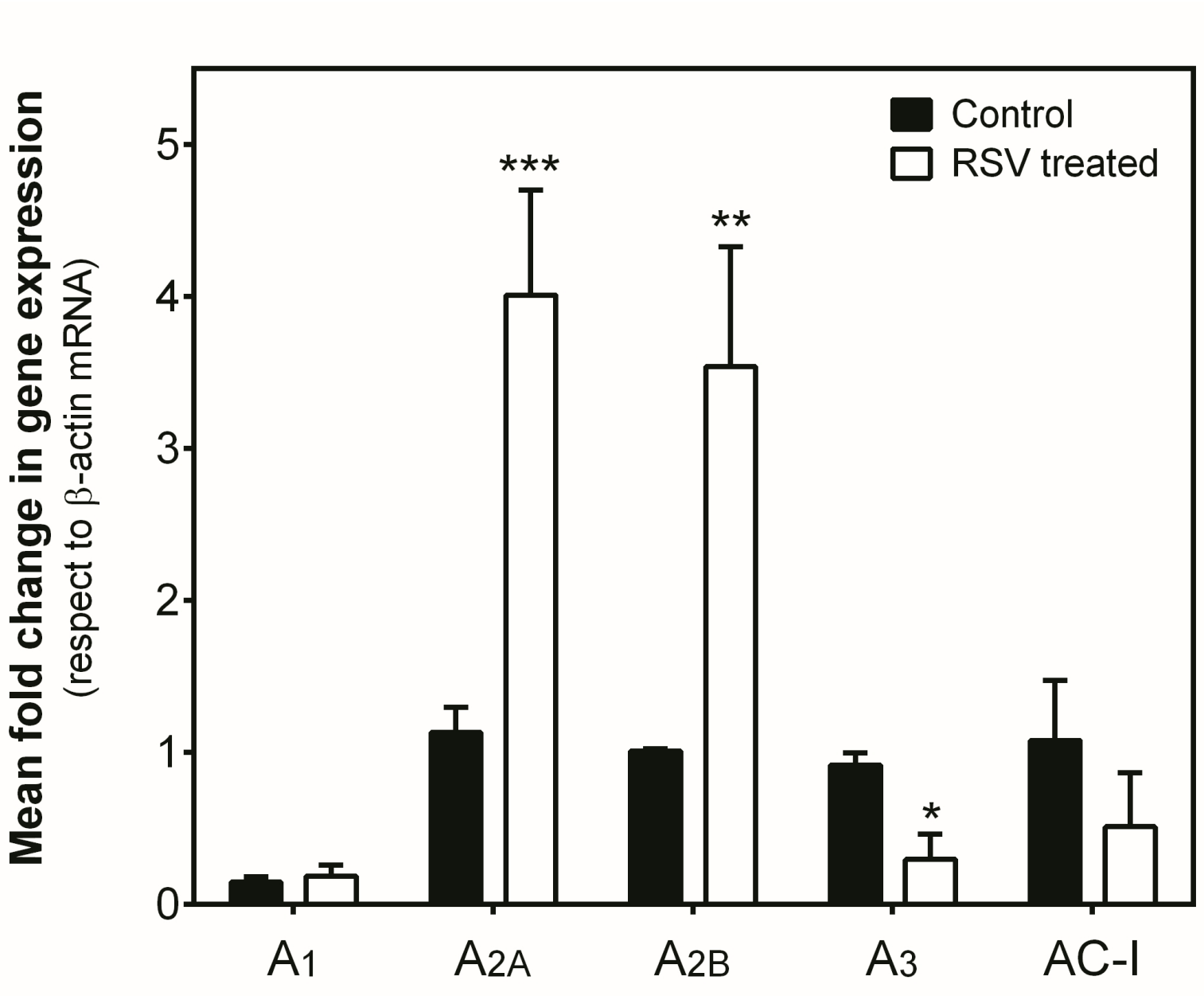
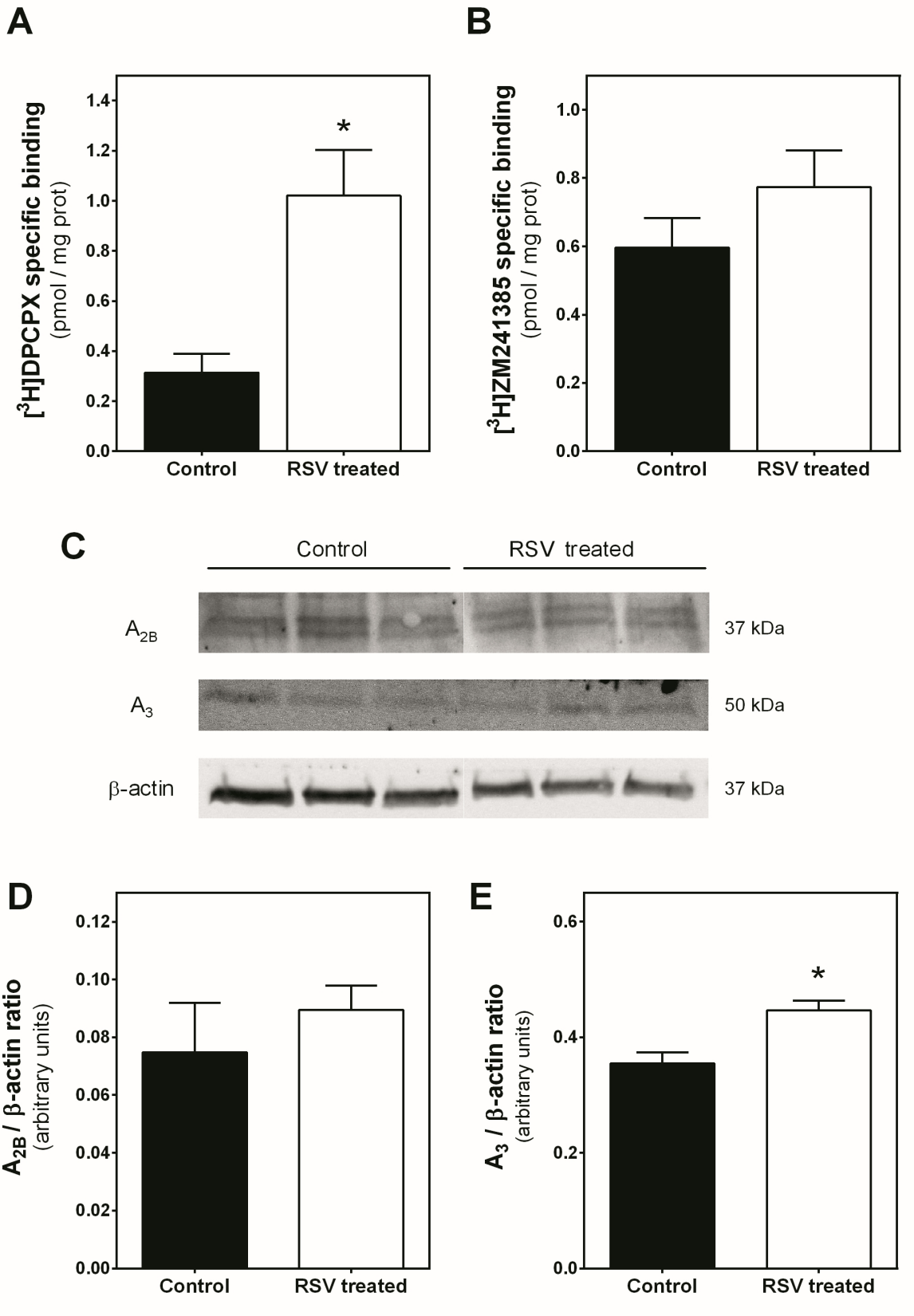
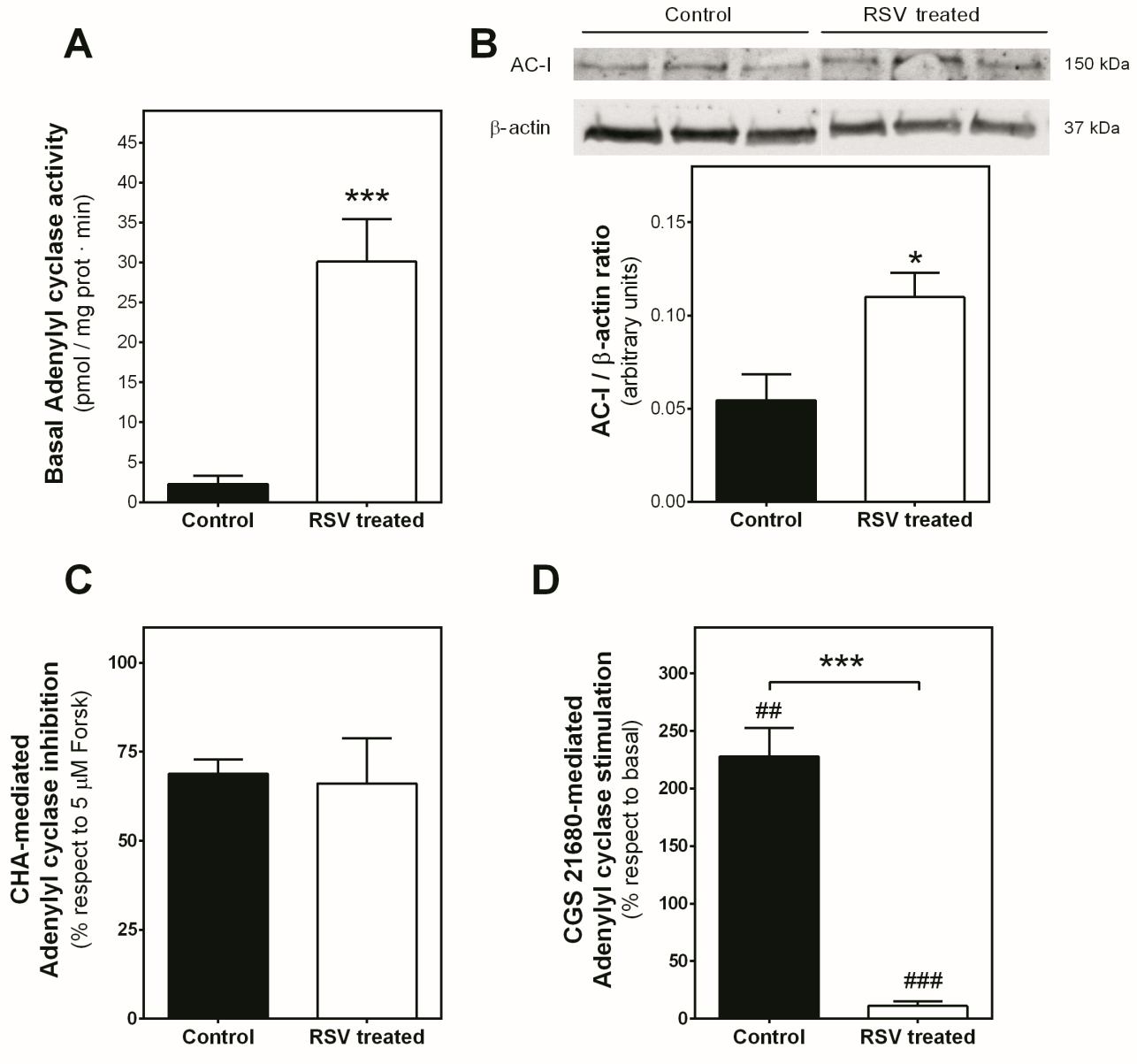
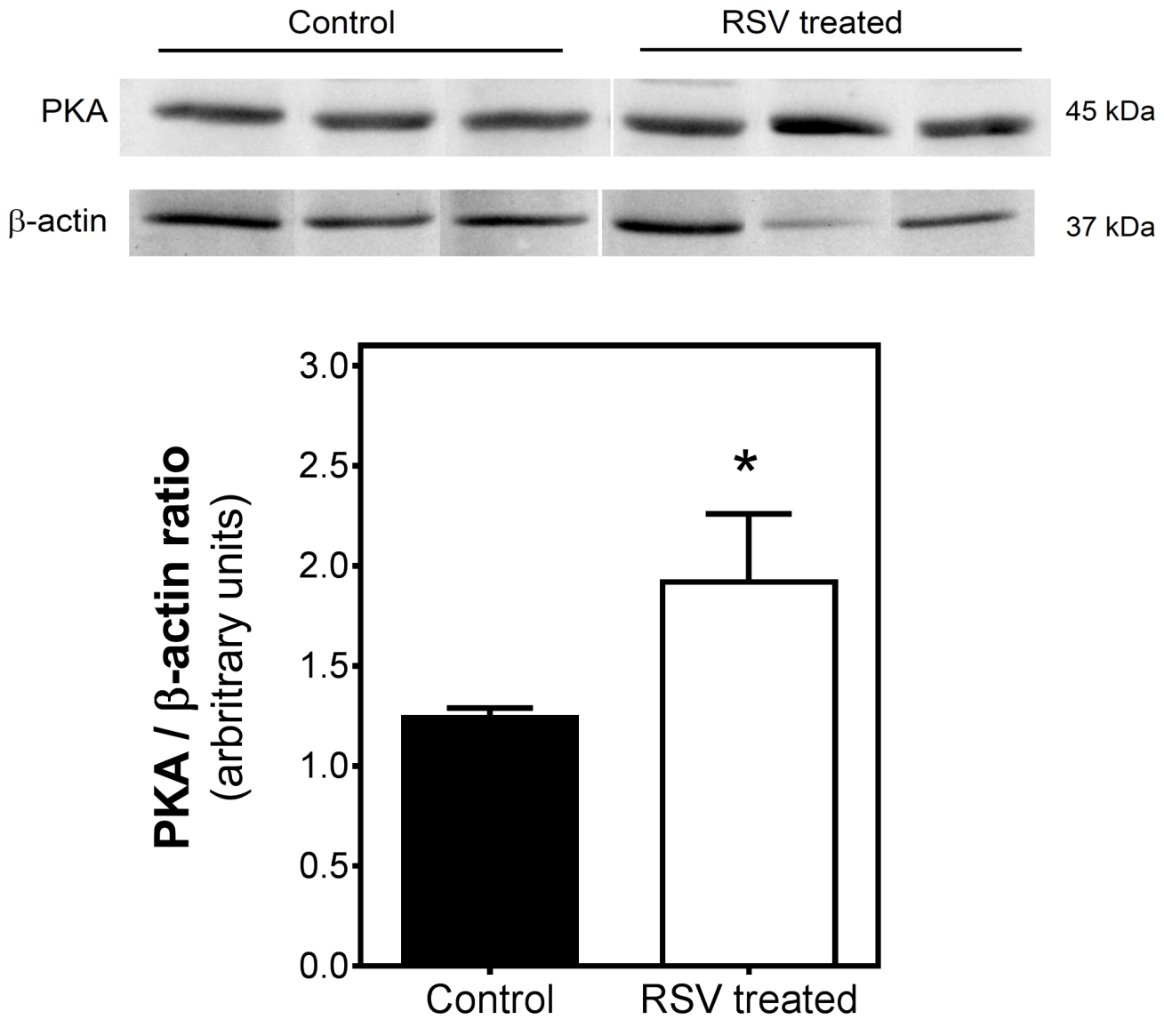
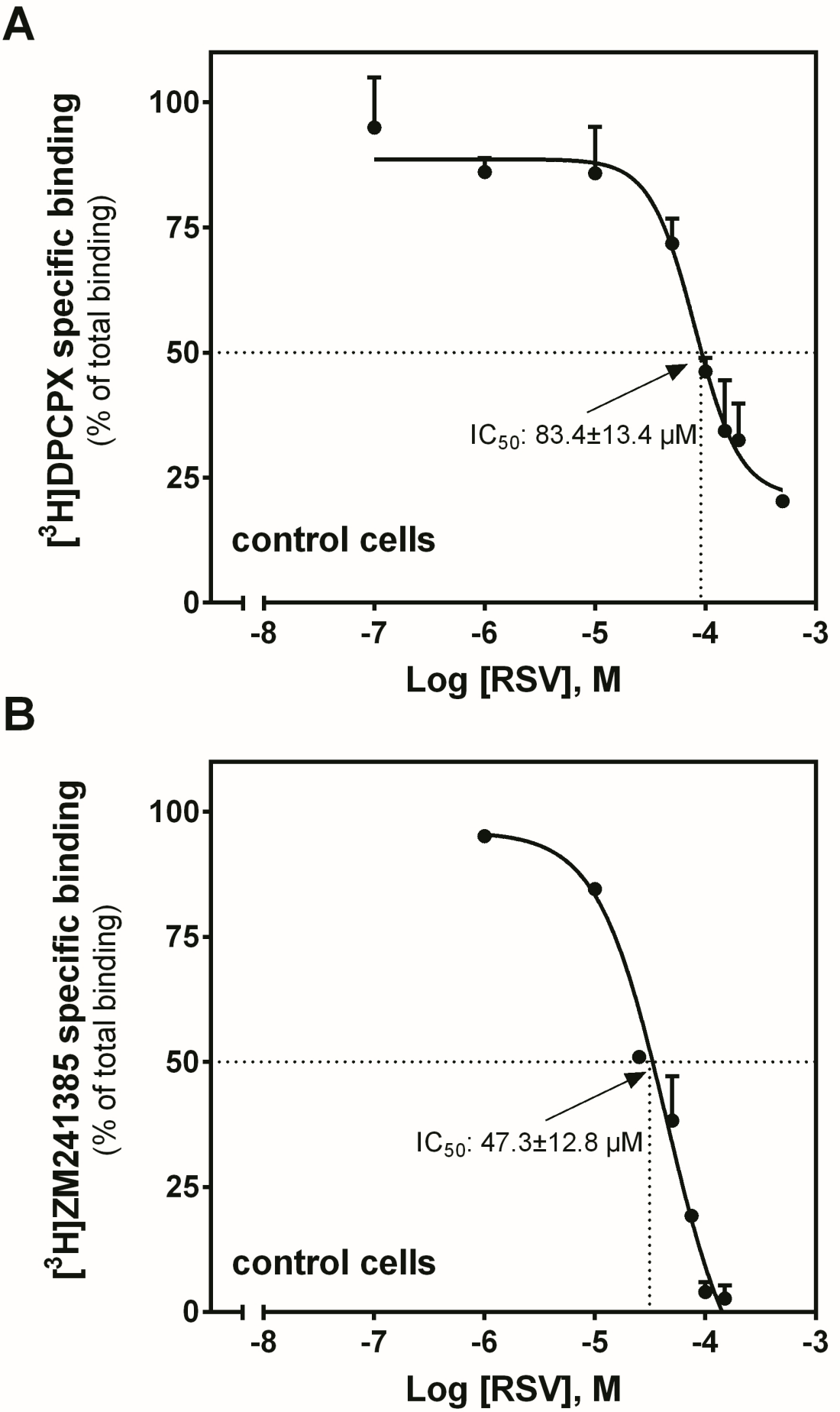
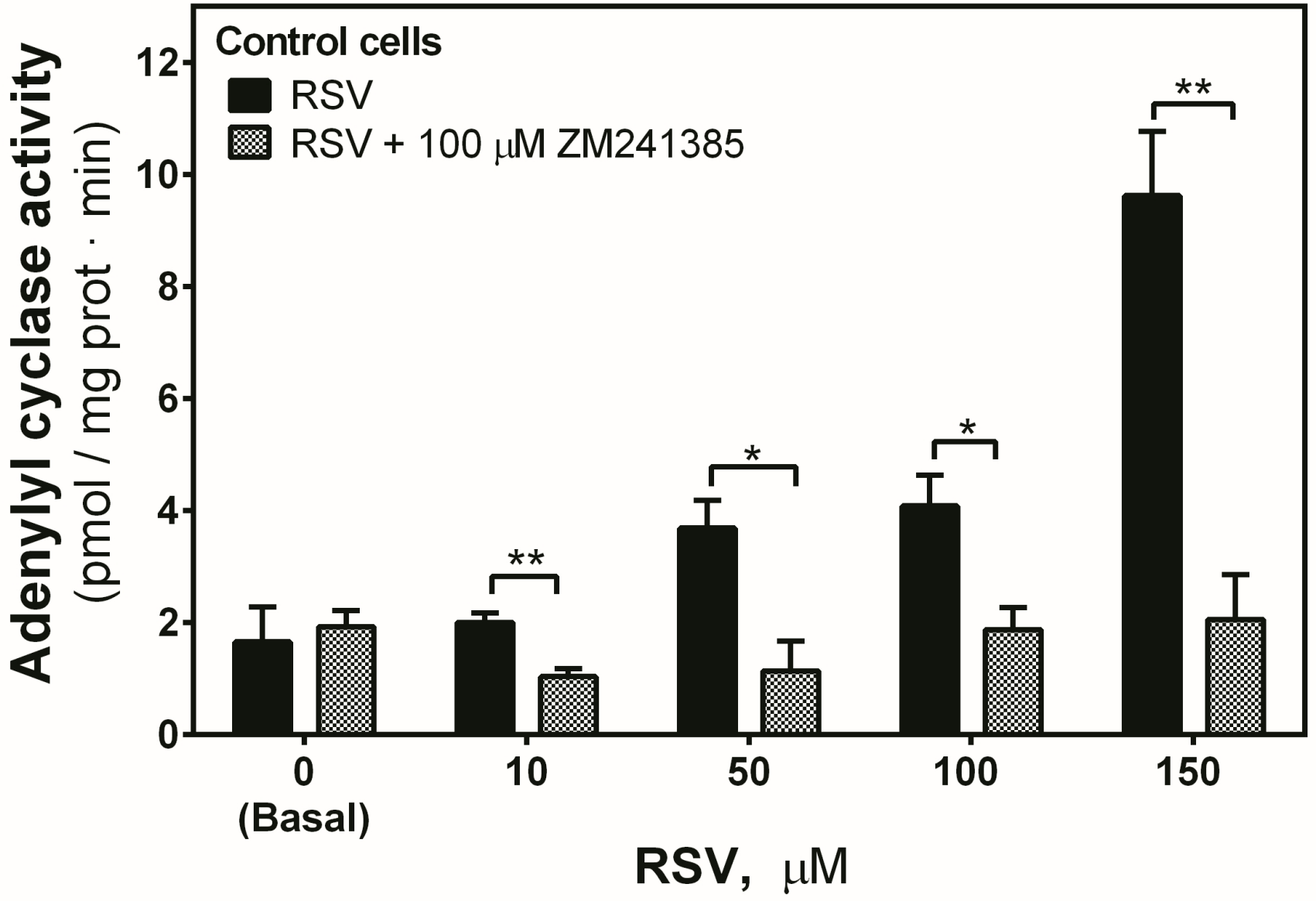

Mean fold change in gene expression
(respect to β-actin mRNA)
Control
RSV treated
0
1
2
3
4
5
***
**
*
A1
A2A
A2B
A3
AC-I

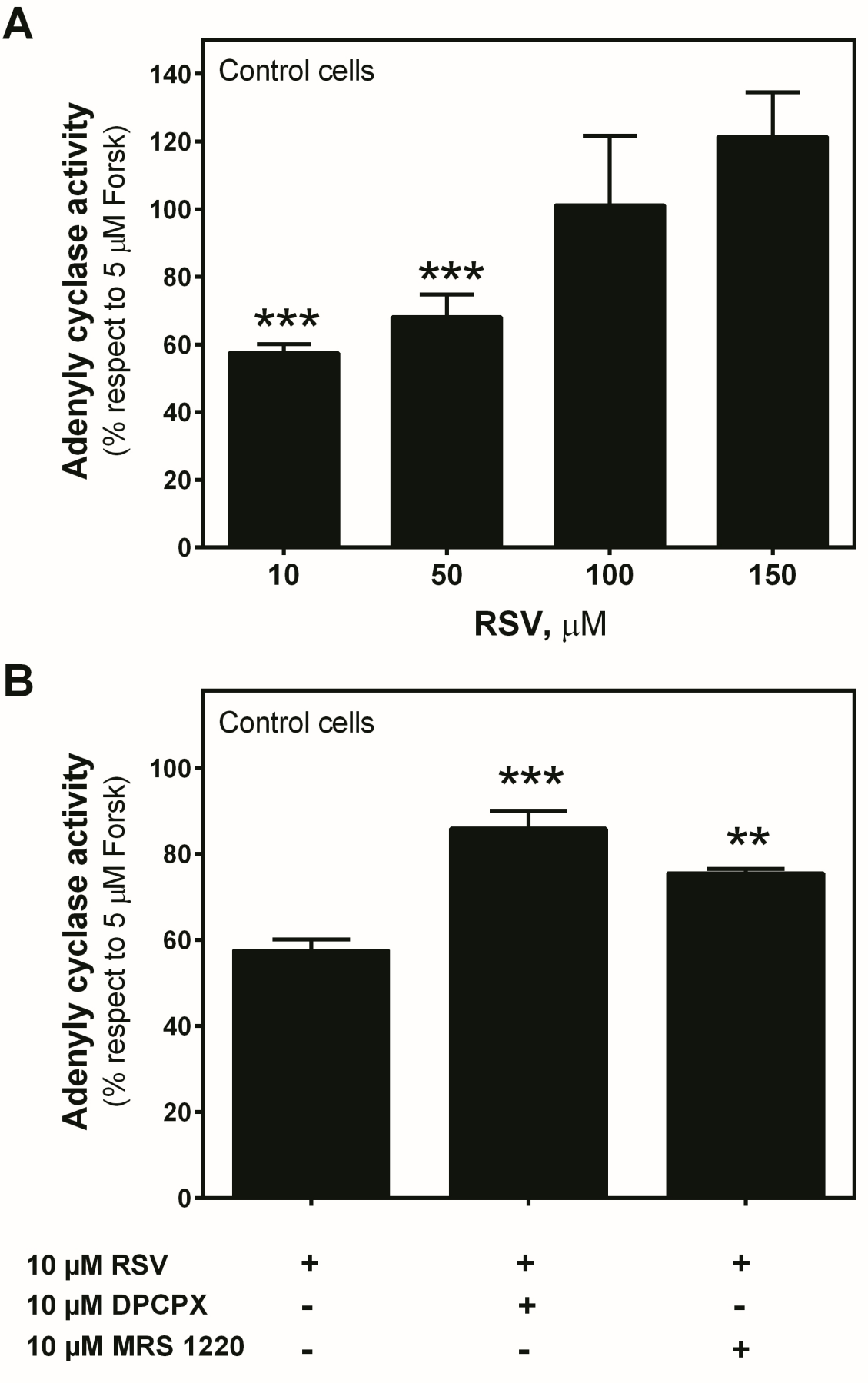

A
[3H]DPCPX specific binding
(pmol / mg prot)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
Control
RSV treated
B
[3H]ZM241385 specific binding
(pmol / mg prot)
1.0
0.8
0.6
0.4
0.2
0.0
Control
RSV treated
C
Control
RSV treated
A2B
37 kDa
A3
50 kDa
β-actin
37 kDa
D
A2B / β-actin ratio
(arbitrary units)
0.12
0.10
0.08
0.06
0.04
0.02
0.00
Control
RSV treated
E
A3 / β-actin ratio
(arbitrary units)
0.6
0.4
0.2
0.0
*
Control
RSV treated

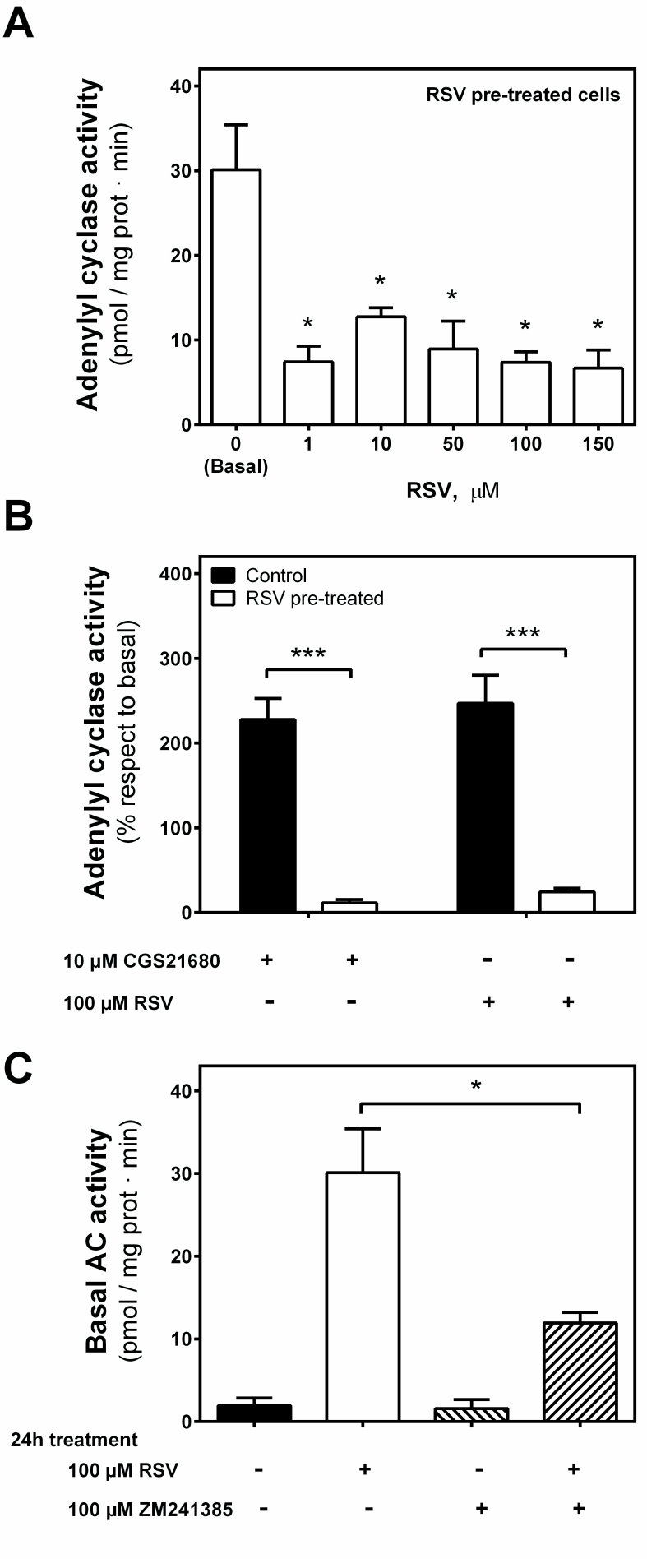

A
Basal Adenylyl cyclase activity
(pmol / mg prot · min)
45
40
35
30
25
20
15
10
5
0
***
Control
RSV treated
B
Control
RSV treated
AC-I
150 kDa
β-actin
37 kDa
AC-I / β-actin ratio
(arbitrary units)
0.15
0.10
0.05
0.00
*
Control
RSV treated
C
CHA-mediated
Adenylyl cyclase inhibition
(% respect to 5 μM Forsk)
100
75
50
25
0
Control
RSV treated
D
CGS 21680-mediated
Adenylyl cyclase stimulation
(% respect to basal)
300
250
200
150
100
50
0
***
##
###
Control
RSV treated

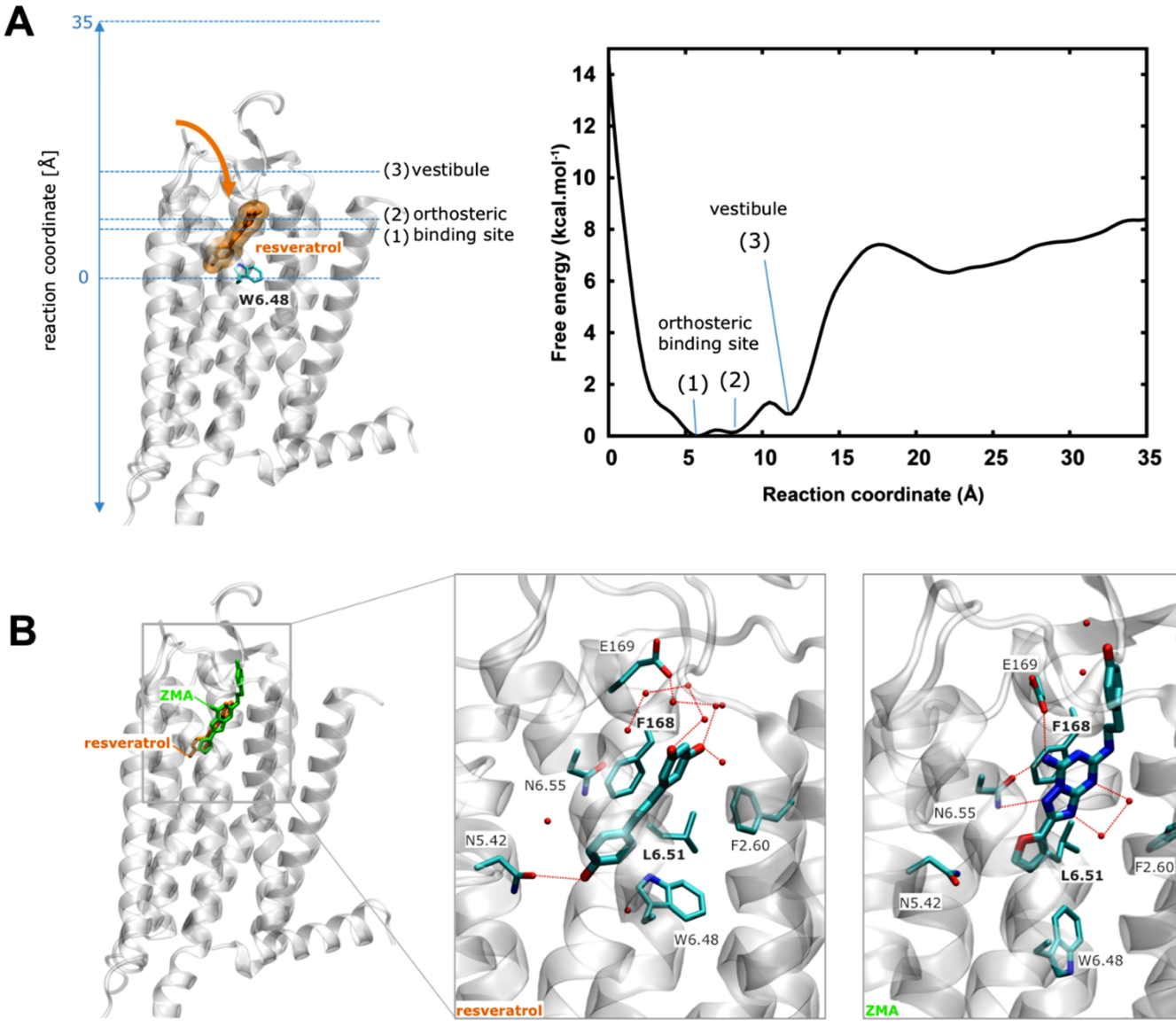

Control
RSV treated
PKA
45 kDa
β-actin
37 kDa
PKA / β-actin ratio
(arbitrary units)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
Control
RSV treated


A
[3H]DPCPX specific binding
(% of total binding)
100
75
50
25
0
IC50: 83.4±13.4 μM
control cells
-8
-7
-6
-5
-4
-3
Log [RSV], M
B
[3H]ZM241385 specific binding
(% of total binding)
100
75
50
25
0
IC50: 47.3±12.8 μM
control cells
-8
-7
-6
-5
-4
-3
Log [RSV], M


Control cells
RSV
RSV + 100 μM ZM241385
Adenylyl cyclase activity
(pmol / mg prot · min)
0
2
4
6
8
10
12
**
*
*
**
0
(Basal)
10
50
100
150
RSV, μM


A
Control cells
Adenyly cyclase activity
(% respect to 5 μM Forsk)
140
120
100
80
60
40
20
0
***
***
10
50
100
150
RSV, μM
B
Control cells
Adenyly cyclase activity
(% respect to 5 μM Forsk)
100
80
60
40
20
0
***
**
10 μM RSV + + +
10 μM DPCPX - + -
10 μM MRS 1220 - - +


A
RSV pre-treated cells
Adenylyl cyclase activity
(pmol / mg prot · min)
40
30
20
10
0
*
*
*
*
*
0
(Basal)
1
10
50
100
150
RSV, μM
B
Control
RSV pre-treated
Adenylyl cyclase activity
(% respect to basal)
400
300
200
100
0
***
***
10 μM CGS21680 + + - -
100 μM RSV - - + +
C
Basal AC activity
(pmol / mg prot · min)
40
30
20
10
0
*
24h treatment
100 μM RSV - + - +
100 μM ZM241385 - - + +


A
35
0
reaction coordinate [Å]
(3) vestibule
(2) orthosteric
(1) binding site
resveratrol
W6.48
Free energy (kcal.mol-1)
Reaction coordinate (Å)
vestibule
(3)
orthosteric
binding site
(1)
(2)
B
ZMA
resveratrol
E169
F168
N6.55
N5.42
L6.51
F2.60
W6.48
resveratrol
ZMA

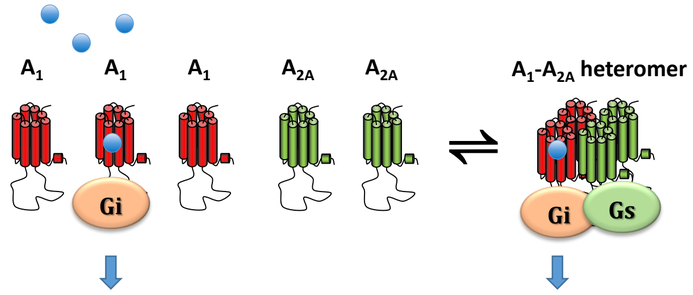
Low

RSV

High

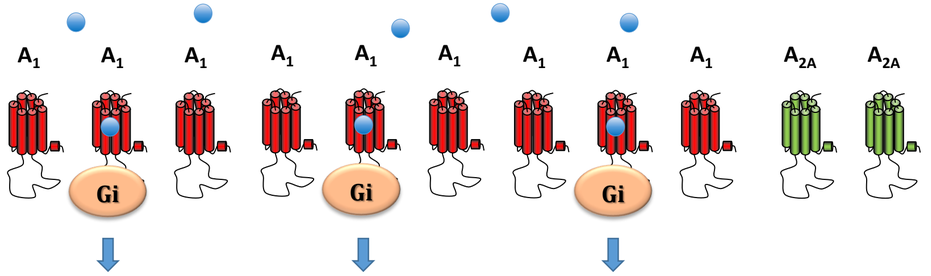
Concentration dependence

Selective A_1 agonist: RSV primarily activates the A_1 receptor due to the higher affinity of RSV for A_1 compared to that for A_{2A}.



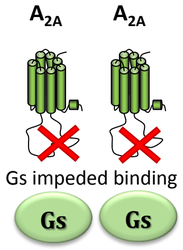
Blocked AC activity

RSV primarily activates the A_1 due to the higher affinity of RSV for A_1 compared to that for A_{2A}. Increased number of A_1 receptors at the cell membrane level with an inhibitory effect on AC activity.



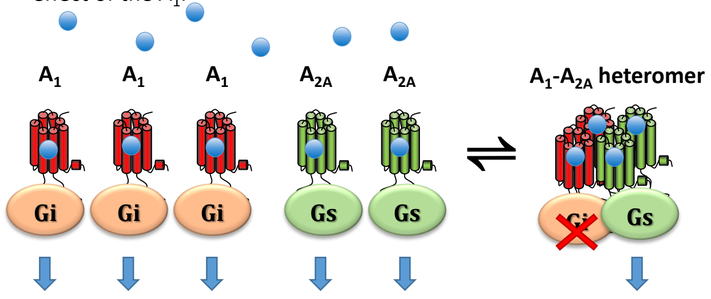
Heteromerization of receptors induces a **Gs/Gi protein switch** for the A_{2A}, leading to an inhibitory effect on AC activity.

A_{2A} inverse agonist: RSV diminishes tonic activation of A_{2A}.



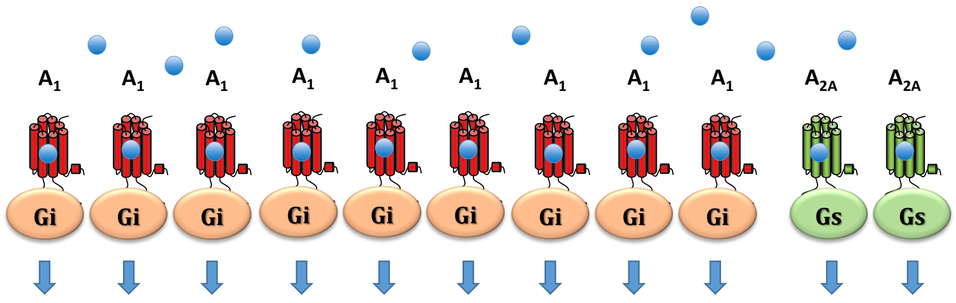
Blocked AC activity

Dual A_1 and A_{2A} agonist: RSV activates both the A_1 and A_{2A} but the stimulating effect of A_{2A} overcompensates the inhibitory effect of the A_1.



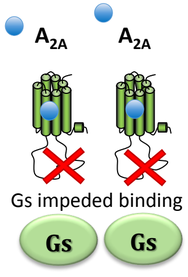
Stimulated AC activity

RSV activates both the A_1 and A_{2A}. The stimulating effect of A_{2A} DOES NOT overcompensate the inhibitory effects of A_1, which are increased after RSV treatment.



Heteromerization of receptors induces a **Gs/Gi protein switch** for the A_{2A}, leading to an inhibitory effect on AC activity.

A_{2A} inverse agonist: RSV diminishes tonic activation of A_{2A}.

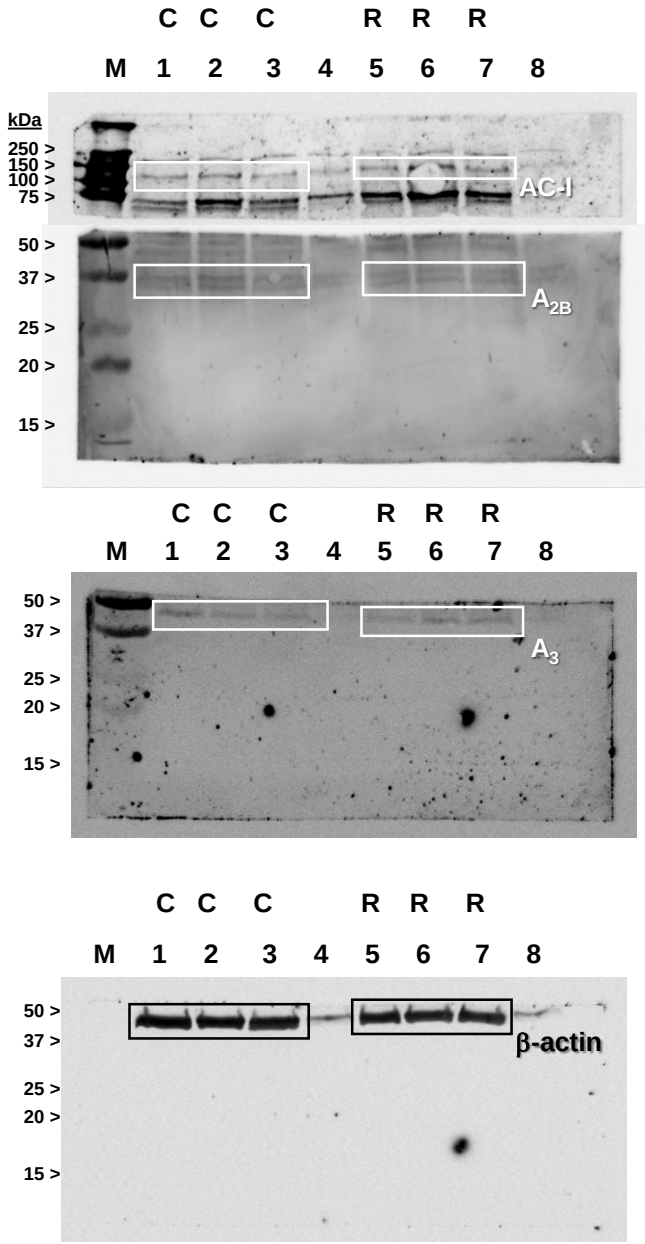


Blocked AC activity

0 h → 24 h

Time dependence of RSV exposure

Supplemental Scheme 1



C, control
R, resveratrol 100 µM

Fig. 3B

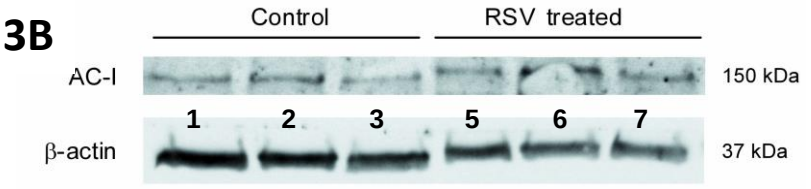
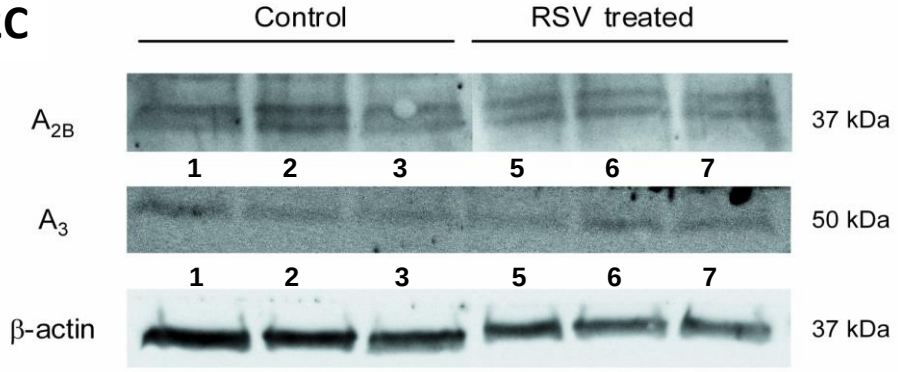


Fig. 2C



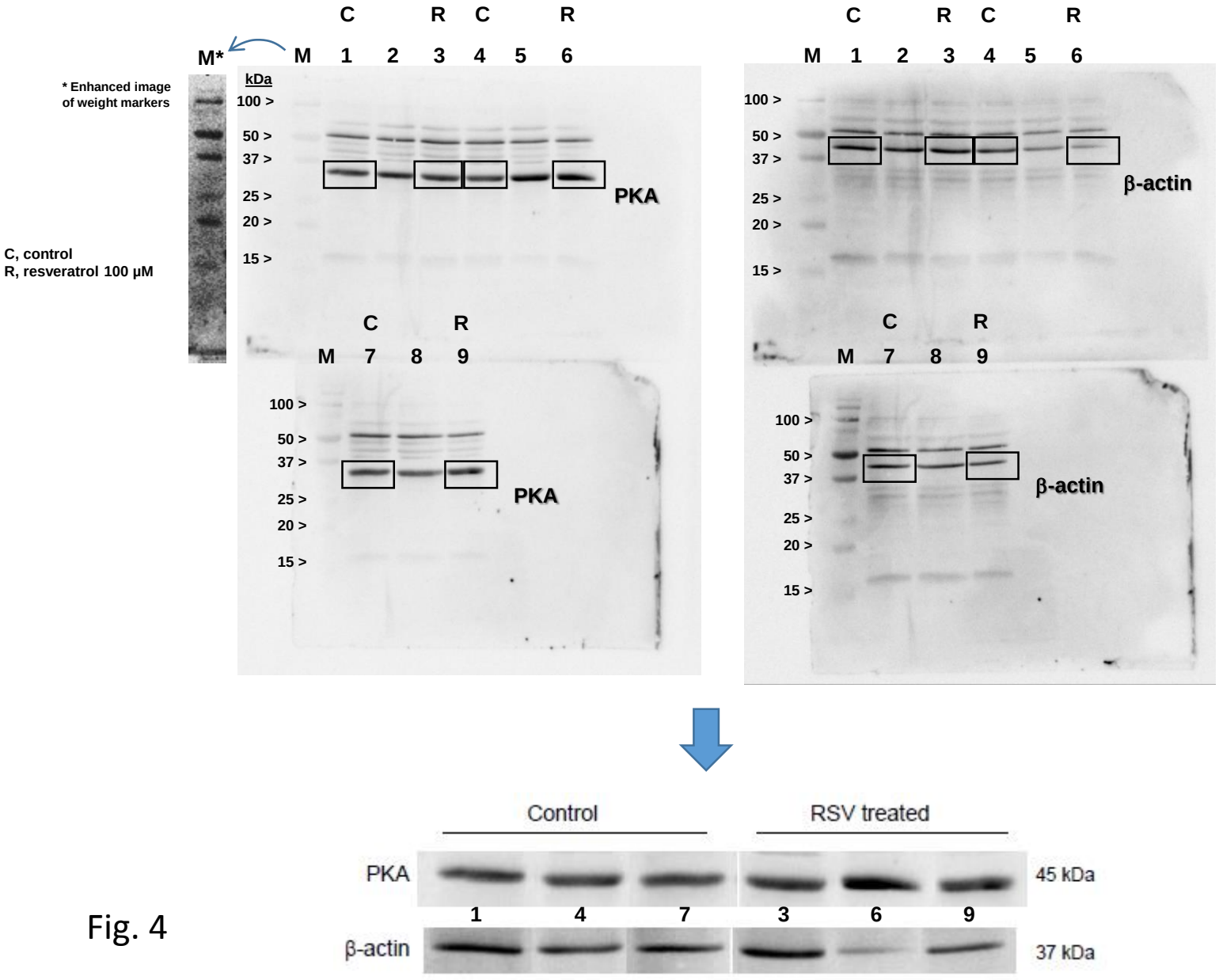

* Enhanced image of weight markers
C, control
R, resveratrol 100 µM
M*
M 1 2 3 4 5 6
C R C R
kDa
100 >
50 >
37 >
25 >
20 >
15 >
PKA
M 7 8 9
C R
β-actin
Control
RSV treated
45 kDa
37 kDa

Fig. 4

SUPPLEMENTAL INFORMATION

THE ANTIOXIDANT RESVERATROL ACTS AS A NON-SELECTIVE ADENOSINE RECEPTOR AGONIST

Sánchez-Melgar A[1,2], Albasanz JL[1,2,3]*, Guixà-González R[4], Saleh N[5], Selent J[6] and Martín M[1,2,3]

SUPPLEMENTAL TABLE 1

Source	PDB ID	Structure Title	Uniprot Acc	Protein Name	Classification	PubMed ID
Arachis hypogaea	1Z1F	Crystal structure of stilbene synthase from Arachis hypogaea (resveratrol-bound form)	Q9SLV5	stilbene synthase	TRANSFERASE	16028220
Bos taurus	2JIZ	The Structure of F1-ATPase inhibited by resveratrol.	P05631	ATP synthase subunit gamma, mitochondrial	HYDROLASE	17698806
Canavalia lineata	4DPN	Crystal Structure of ConM Complexed with Resveratrol	P81460	Concanavalin-A	METAL BINDING PROTEIN	
Daboia russelii	4QER	Crystal Structure of the Complex of Phospholipase A2 with Resveratrol at 1.20 A Resolution	D0VX11	Phospholipase A2 VRV-PL-VIIIa	HYDROLASE/HYDROLASE INHIBITOR	25541253
Dictyostelium discoideum	3MNQ	Crystal structure of myosin-2 motor domain in complex with ADP-metavanadate and resveratrol	P08799	Myosin-2 heavy chain	MOTOR PROTEIN/INHIBITOR	
Homo sapiens	1DVS	CRYSTAL STRUCTURE OF HUMAN TRANSTHYRETIN IN COMPLEX WITH RESVERATROL	P02766	Transthyretin	HORMONE/GROWTH FACTOR	10742177
	1SG0	Crystal structure analysis of QR2 in complex with resveratrol	P16083	Ribosyldihydronicotinamide dehydrogenase [quinone]	OXIDOREDUCTASE	15350128
	2L98	Structure of trans-Resveratrol in complex with the cardiac regulatory protein Troponin C	P63316	Troponin C, slow skeletal and cardiac muscles	CONTRACTILE PROTEIN	21226534
	2YDX	Crystal structure of human S-adenosylmethionine synthetase 2, beta subunit	Q9NZL9	Methionine adenosyltransferase 2 subunit beta	OXIDOREDUCTASE	23425511
	3CKL	Crystal structure of human cytosolic sulfotransferase SULT1B1 in complex with PAP and resveratrol	O43704	Sulfotransferase family cytosolic 1B member 1	TRANSFERASE	
	3FTS	Leukotriene A4 hydrolase in complex with resveratrol	P09960	Leukotriene A-4 hydrolase	HYDROLASE	19618939
	4HDA	Crystal structure of human Sirt5 in complex with Fluor-de-Lys peptide and resveratrol	Q9NXA8	NAD-dependent protein deacylase sirtuin-5, mitochondrial	HYDROLASE/HYDROLASE ACTIVATOR	23185430
	4JAZ	Crystal structure of the complex between PPARgamma LBD and trans-resveratrol	P37231	Peroxisome proliferator-activated receptor gamma	TRANSCRIPTION REGULATION	24796862

	4PP6	Crystal Structure of the Estrogen Receptor alpha Ligand-binding Domain in Complex with Resveratrol	P03372	Estrogen receptor	PROTEIN BINDING	24771768
	4Q93	Crystal structure of resveratrol bound human tyrosyl tRNA synthetase	P54577	Tyrosine--tRNA ligase, cytoplasmic	LIGASE	25533949
	4QOH	Crystal structure of fad quinone reductase 2 in complex with resveratrol at 1.6A	P16083	Ribosyldihydronicotinamide dehydrogenase [quinone]	OXIDOREDUCTASE	
	4QOJ	CRYSTAL STRUCTURE OF FMN QUINONE REDUCTASE 2 IN COMPLEX WITH RESVERATROL AT 1.85A	P16083	Ribosyldihydronicotinamide dehydrogenase [quinone]	OXIDOREDUCTASE	
	5CR1	Crystal structure of TTR/resveratrol/T4 complex	P02766	Transthyretin	TRANSPORT PROTEIN	26468275
Medicago sativa	1CGZ	CHALCONE SYNTHASE FROM ALFALFA COMPLEXED WITH RESVERATROL	P30074	Chalcone synthase 2	TRANSFERASE	10426957
	1U0W	An Aldol Switch Discovered in Stilbene Synthases Mediates Cyclization Specificity of Type III Polyketide Synthases: 18xCHS+resveratrol Structure	P30074	Chalcone synthase 2	TRANSFERASE	15380179
Neurospora crassa	5U90	Crystal structure of Co-CAO1 in complex with resveratrol	Q7S860	Carotenoid oxygenase 0	OXIDOREDUCTASE	28493664
Novosphingobium aromaticivorans	5J54	The Structure and Mechanism of NOV1, a Resveratrol-Cleaving Dioxygenase	Q2GA76	Carotenoid oxygenase	OXIDOREDUCTASE	27911781
synthetic construct	5BTR	Crystal structure of SIRT1 in complex with resveratrol and an AMC-containing peptide		AMC-containing peptide	HYDROLASE/SUBSTRATE	26109052

Supplemental Table 1. Available 3D models of RSV interacting proteins. Several proteins have been crystallized in complex with RSV and modeled in 3D. PDB ID, Protein Data Bank ID (https://www.wwpdb.org/); PubMedID, PubMed unique identifier (https://www.ncbi.nlm.nih.gov/pubmed/); Uniprot Acc, Uniprot accession number (http://www.uniprot.org/).

Supplemental Movie S1. Extracellular access of RSV into the A_{2A} receptor. Initial metadynamics trajectory showing the interaction between one RSV molecule (cyan/red sticks and orange transparent surface) and the extracellular surface of the A_{2A} receptor (grey surface) and subsequent access into the orthosteric cavity.

Supplemental Movie S2. RSV binding at the orthosteric binding pocket. Initial metadynamics trajectory of RSV (cyan/red spheres) binding to the A_{2A} receptor (light grey cartoon). Interacting residues roughly located at binding site 1 (energy minima 1 in Figure 9A) are highlighted by a transparent red surface. Those that are only involved in binding site 2 (energy minima 2 in Figure 9A) are highlighted by a transparent orange surface.