Reengineering the collision coupling and diffusion mode of the $\text{A}_2\text{A}$-adenosine receptor: palmitoylation in helix 8 relieves confinement

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Running title: Introducing a palmitoylation site into the $\text{A}_2\text{A}$-receptor

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Background: The $\text{A}_2\text{A}$-receptor engages $\text{G}_s$ by restricted collision coupling and lacks a palmitoyl moiety in its C-terminus.

Result: Engineering palmitoylated cysteine into the C-terminus relieved restricted collision coupling and resulted in accelerated diffusion of the agonist-liganded $\text{A}_2\text{A}$-receptor.

Conclusion: Restricted collision coupling arises from limits imposed on receptor diffusion.

Significance: Agonist induced confinement of the $\text{A}_2\text{A}$-receptor in a structure consistent with a lipid raft.

SUMMARY

The $\text{A}_2\text{A}$-adenosine receptor undergoes restricted collision coupling with its cognate $\text{G}$ protein $\text{G}_s$ and lacks a palmitoylation site at the end of helix 8 in its intracellular carboxyl terminus. We explored the hypothesis that there was a causal link between the absence of a palmitoyl moiety and restricted collision coupling by introducing a palmitoylation site. The resulting mutant $\text{A}_2\text{A}$-receptor-R$^{309}\text{C}$ underwent palmitoylation as verified by both, mass spectrometry and metabolic labeling. In contrast to the wild type $\text{A}_2\text{A}$-receptor, the concentration-response curve for agonist-induced cAMP accumulation was shifted to the left with increasing expression levels of $\text{A}_2\text{A}$-receptor-R$^{309}\text{C}$, an observation consistent with collision coupling. Single particle tracking of quantum dot-labeled receptors confirmed that wild type and mutant $\text{A}_2\text{A}$-receptor differed in diffusivity and diffusion mode: agonist activation resulted in a decline in mean square displacement of both receptors, but the drop was substantially more pronounced for the wild type receptor. In addition, in the agonist-bound state, the wild type receptor was frequently subject to confinement events (estimated radius 110 nm). These were rarely seen with the palmitoylated $\text{A}_2\text{A}$-receptor-R$^{309}\text{C}$, the preferred diffusion mode of which was a random walk in both, the basal and the agonist-activated state. Taken together, the observations link restricted collision coupling to diffusion limits imposed by the absence of a palmitoyl moiety in the C-terminus of the $\text{A}_2\text{A}$-receptor. The experiments allowed for visualizing local confinement of an agonist-activated $\text{G}$ protein-coupled receptor in an area consistent with the dimensions of a lipid raft.

The $\text{A}_2\text{A}$-adenosine receptor is of interest because of several reasons: (i) it is among the most frequently blocked pharmacological targets, because it is the site of action of caffeine (1). In fact, in prospective studies blockage of the $\text{A}_2\text{A}$-receptor by caffeine consumption reduces the risk of developing Parkinson’s disease (2). Accordingly, the $\text{A}_2\text{A}$-selective antagonist istradefylline has entered phase III clinical trials (3,4). The efficacy of istradefylline is remarkable, because it is first non-dopaminergic compound shown to be active in Parkinson’s disease. Conversely, topical preparations of $\text{A}_2\text{A}$-selective agonists are tested in clinical trials, e.g., for promotion of dermal wound healing and for suppression of asthma (5,6); the underlying rationale being the observation that $\text{A}_2\text{A}$-receptor activation stimulates endothelial cell proliferation (7,8) and suppresses proinflammatory signals in macrophages and T-cells (9), respectively. (ii) When compared to other $\text{GPCRs}$ of the class A (rhodopsin-like) $\text{G}$ protein-coupled receptors (GPCRs), the $\text{A}_2\text{A}$-
receptor has a long C-terminus that provides a docking site for several proteins, which direct the fate of the receptor from its site of synthesis in the endoplasmic reticulum to its lysosomal degradation (10). In addition, the long C-terminus also allows for recruitment of additional signaling molecules (11, 12). (iii) The structure of the A2A-receptor is understood at atomic resolution, in both, the antagonist- (13) and the agonist-bound state (14, 15). Structure-based molecular dynamics simulations suggest that the A2A-receptor may have a cholesterol binding site that promotes agonist binding; this may allow the receptor to sample the membrane environment (16). In fact, cholesterol depletion precludes coupling of the receptor to Gs, but does not impede its ability to recruit ARNO and to activate ERK-phosphorylation (17). (iv) The vast majority of GPCRs carry one or several cysteine residues in their C-terminus some 20 amino acids removed from the end of transmembrane helix 7. The cysteine residue(s) is/are subject to palmitoylation. This modification is thought to anchor the amphipathic helix 8, which runs parallel to the membrane plane, and stabilize it (18). In the A2A-receptor there isn’t any cysteine in this region. This is also true for the chemokine receptor CXCR4 (the receptor for stromal cell derived factor/CXCL12). In contrast to CXCR4, in which helix 8 only comprises a single turn followed by an extended flexible segment (19), helix 8 in the A2A-receptor has four helical turns and this is seen in, both the antagonist- and the agonist-bound state (13-15). It is therefore not clear, why the A2A-receptor should have evolved to lack the palmitoylation site. (iv) The A2A-receptor has long been known to differ from other Gs-coupled receptors by its G protein-coupling mode (20): when examined in the same cell (i.e., the turkey erythrocyte membrane), the β-adrenergic receptor has access to all Gs moieties, while the A2A-receptor can only promote activation of a limited number of available Gs molecules. This coupling mode was termed restricted collision coupling (20, 21). Restricted collision coupling is not a unique property of the turkey erythrocyte membrane. It can also be observed in mammalian cells, where the A2A-receptor is expressed endogenously, e.g., the human platelet (22).

In the present work, we explored the hypothesis that there is a link between restricted collision coupling and the absence of a palmitoyl-moiety in the C-terminus of the A2A-receptor, because its absence limited the membrane area that was visited by the agonist-activated A2A-receptor. In fact, engineering a palmitoylation site into the A2A-receptor eliminated restricted collision coupling, accelerated diffusion of the quantum dot-labeled receptor and eliminated its confinement that was seen in the agonist-bound state.

Materials and Methods

Materials: All cell culture reagents and Lipofectamine Plus™ were from Invitrogen (Carlsbad, CA) except fetal calf serum (FCS; PAA Laboratories; Pasching, Austria), [2,8-3H]adenine, (28.1 Ci/mmol) and [9,10-3H(N)]palmitic (47.7 Ci/mmol) acid were purchased from PerkinElmer (Boston, MA), [3H]ZM241385 (specific activity 50 Ci/mmol) from American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA), unlabeled ZM241385 from Tocris Bioscience (Bristol, UK), quantum dots Q11022MP Qdot® 655 goat F(ab′)2 anti-mouse IgG conjugate (H+L) from Molecular Probes/Invitrogen (Grand Island, NY, USA), the mouse monoclonal anti-FLAG® M2 antibody and casein from bovine milk were from Sigma (St. Louis, MO, USA). Polyclonal antibodies recognizing ERK1/2 (p44/42 MAP kinase) and its dually phosphorylated (active) form were purchased from Cell Signalling Technology (Danvers, MA, USA), the mouse monoclonal antibody against the A2A-adenosine receptor (clone 7F6-G6-A2) from Millipore (Billerica, MA, USA), the rabbit polyclonal antibody against flotillin-1 was from Sigma (St. Louis, MO, USA), the rabbit polyclonal antibody against CXCR4 (the receptor for stromal cell derived factor/CXCL12) from Abcam Plc (Cambridge, UK), the rabbit antiserum directed against Gβ6 was raised against amino acids 8-23 of Gβ1/2 (23). Protein A-sepharose, anti-mouse and anti-rabbit IgG1 (HRP) horseradish were from GE Healthcare (Little Chalfont, UK). Complete protease inhibitor cocktail was from Roche (Mannheim, Germany). The source of the other reagents and chemicals has been listed previously (17).

Cell transfection and cell culture: Arginine 309 was replaced by cysteine in the human A2A-receptor cDNA using the QuikChange Lightning site-directed mutagenesis kit (Stratagene, Carlsbad, CA, USA). The forward primer sequence of the forward primer sequence was as follows (5′ to 3′ direction with the mutated nucleotides indicated in bold and underlined to introduce the R309C mutation and a silent mutation for insertion of an Eco72I/PmaCI site, respectively): C ATT CGC AGC CAC GTG

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CTG TGC CAG CAA GAA CCT TTC. Several versions of the receptor cDNA were prepared, namely one encoding wild type and mutant receptors tagged on their C-terminus with YFP. The sequence was confirmed by automated DNA sequencing. Human embryonic kidney (HEK) 293 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing high glucose (4.5 g/L = 25 mM), 10% FCS and 2 mM glutamine at 37°C in a 5% CO2 humidified atmosphere. For transient expression, HEK293 cells were transfected with plasmids encoding wild type or mutant A2A-receptor using the calcium phosphate precipitation method. These cells were used 24 to 48 h later for experiments. For the generation of stable cell lines, transfected cells were subjected to selection in the presence of geneticin (G418; 0.5 to 0.75 g/L). After 7-14 days single colonies emerged, which were expanded and maintained in the presence of 0.25 g/L G418. In some instances, HEK293 cells expressing an YFP-tagged A1-receptor (24) were used as a control. Receptor expression was visualized by confocal microscopy on a Zeiss Axiovert 200-LSM 510 microscope equipped with argon and helium/neon lasers (30 and 1 mW, respectively) and a 63x oil immersion objective (Zeiss Plan-Neofluar) as in (17). Stable HEK293 cell lines expressing FLAG-tagged wild type A2A-receptor and A2A-receptor-R309C were produced by retroviral infection using pBABEpuro. The retroviruses were produced in Phoenix ecotropic cells. Infected cells were subjected to selection with puromycin (2 µg/ml).

Cyclic AMP accumulation, radioligand binding and ERK phosphorylation- The conditions for determining agonist-stimulated cAMP accumulation are outlined in ref. 24. In brief, cells (3*10^5/well) were seeded into poly-D-lysine-coated 6-well culture plates. On the following day, the cellular ATP pool was metabolically labeled by incubating cells for 16 h with [3H]adenine (1 µCi/well). Cells were starved by removing FCS from the culture medium and the cells were subsequently incubated for 1 h in the presence of the phosphodiesterase inhibitor Ro-20-1724 (100 µM) and adenosine deaminase (5 µg/mL). The formation of cAMP was stimulated by addition of different concentrations of CGS21680 for 20 min at 37°C. Agonist-stimulated ERK phosphorylation was assessed as in ref. 26. Samples of cellular lysate (20 to 30 µg protein) were electrophoretically resolved and transferred to a nitrocellulose membrane. Activation of MAPK was detected using an anti-phospho-ERK1/2 antibody, anti-holo ERK1/2 antibody served as loading control. Immunoreactive bands were visualized using a FluorChemHD2 imaging system (Alpha Innotech Corp., San Leandro, CA, USA) and quantified using ImageJ (Version 1.45r). Binding assays were carried out with the high-affinity, A2A-selective antagonist [3H]ZM241385 in a final volume of 0.2 mL containing 50 mM Tris.HCl, pH 7.5, 5 mM MgCl2, 1 mM EDTA, 5 µg/mL adenosine deaminase and additional reagents as outlined in the figure legends. Reaction conditions were as described in ref. 17 except that membranes were used instead of intact cells. These membranes were prepared by resuspending HEK293 cells stably expressing wild type and mutated receptors in buffer containing 25 mM HEPES.NaOH, pH 7.5, 2 mM MgCl2, 1 mM EDTA and subjecting the suspension to two freeze-thaw cycles followed by sonication (four times for 10 s in a ice-cold water bath). Membranes were harvested by centrifugation at 34,000 g for 30 min. The pellet was resuspended in the buffer described above. The protein concentration was estimated by coomassie blue binding and adjusted in the binding reaction (5 to 100 µg/assay) to avoid radioligand depletion.

Metabolic labeling with [3H]palmitate and immunoprecipitation- Labeling was done as described by (27) with the following modifications: cells (3*10^5/well) were seeded into 6-well plates and allowed to adhere. After 20 h, the cells were lipofected with plasmids encoding FLAG-tagged versions of wild type A2A-receptor, A1-receptor or A2A-receptor-R309C. After 4 h, the medium was replaced and cells were grown for another 24 h. Cells were preincubated in serum-free medium supplemented with 10 mg/ml fatty-acid free bovine serum albumin, then labeled with [3H]palmitate (0.25 mCi/well) for 4 h, washed with phosphate buffered saline and resuspended in 0.15 ml buffer (20 mM Tris-HCl pH 7.4, 1 mM EDTA, 100 mM NaCl, 1 % SDS). After 5 min, triton-X100 containing buffer (20 mM Tris-HCl pH 7.4, 1 mM EDTA, 100 mM NaCl, 1 % triton X-100) was added to dilute the SDS concentration to 0.1%. The sample was centrifuged at 16,000 g for 10 min and the resulting supernatant incubated overnight with anti-GFP antibody (2 µl corresponding to 10 µg total IgG) under gentle rotation at 4°C. Subsequently, pre-equilibrated protein A-Sepharose (6 mg per sample) was added and incubated with gentle rotation at 4°C for 3 h.
Samples were washed thrice with triton-X100 containing lysis buffer and eluted by heating the samples in 0.1 mL loading buffer (62.5 mM Tris-HCl pH 6.8, 10 % glycerol, 2 % SDS, 0.001 % bromophenol blue) at 95°C for 2 min. An aliquot of the sample (40 μL) was analyzed by SDS-PAGE, the gel was fixed for 30 min in fixing solution (isopropanol:water:acetic acid = 25:65:10) incubated in NAP100 (GE Healthcare) for 30 min, dried and then exposed to X-ray film (Kodak BioMax MS) at -80°C for 2 - 8 weeks.

Nano LC-MS/MS and multiple reaction monitoring (MRM) mass spectrometry- Membranes were prepared from cells expressing FLAG-tagged versions of wild type A2A-receptor and A2A-receptor-R<sup>309C</sup> as outlined above. Membranes (1 mg/mL) were solubilized in buffer containing 25 mM HEPES.NaOH, pH 7.5 and 0.2 mM dodecyl-β-maltoside. The unsolubilized material was removed by ultracentrifugation (250,000 g for 2 h at 4°C) and the resulting supernatant was incubated with pre-equilibrated anti-FLAG M2 agarose beads for 1 h at 4°C. Beads were transferred to a gravity flow column and washed three times with 0.5 mL solubilization buffer containing 1 % triton-X100. The column was incubated twice with 0.1 mL buffer containing Tris-HCl (20 mM, pH 8) and 6 M urea buffer for 10 min at 25°C under stopped-flow conditions. The eluate was collected by spinning the column in a tabletop ultracentrifuge (30 x g for 1 min). The eluate was transferred to a Microcon ultracel YM-3 column (Millipore GmbH, Austria) and concentrated by centrifugation. The desalted and concentrated sample was digested with trypsin for 12-16 h at 37°C. Concentrated acetic acid was added to stop the digestion reaction. Digested samples were analyzed by nano LC-MS/MS using information-dependent acquisition (IDA) for mapping of tryptic A<sub>2A</sub>-receptor peptides and multiple reaction monitoring (MRM) mass spectrometry to identify the palmitoylated peptide. A Dionex Ultimate 3000 nano HPLC system (Dionex, Amsterdam, The Netherlands) in pre-concentration mode was used for reversed-phase separation of the samples prior to MS using a Dionex Acclaim PepMap 100 C<sub>18</sub> 100 Å, column (150 mm x 75 μm) with a particle size of 3 μm. The mobile phase consisted of solvent A (0.1% aqueous formic acid) and solvent B (80/20 acetonitrile/0.08% aqueous formic acid). Tryptic peptides were eluted using a gradient of 4-60% solvent B in 40 min at a flow rate of 300 nL/min. The eluate was directly introduced into a hybrid triple quadrupole/linear ion trap 4000 QTRAP MS/MS system (AB Sciex, Massachusetts, USA) equipped with a NanoSprayII ion source and was operated with Analyst 1.5.2 software. The experiments were performed in positive ionization mode. Nebulizer gas, ion spray voltage and declustering potential were optimized for each experiment. IDA (information-dependent acquisition) experiments for tryptic peptide identification of the receptor included enhanced MS (EMS) to examine the molecular weight of peptide ions and enhanced resolution (ER) scans to confirm charge states of the ions. Detection of ions above threshold with a charge state of + 2 to +5 or with an unknown charge state triggered collision-induced dissociation of three enhanced product ion scans (EPI) to collate fragment ions and present the product ion spectrum for subsequent database searches. Database searching was carried out using the ProteinPilot™ software (Version 4.0) and the Paragon algorithm (AB Sciex, Massachusetts, USA). MRM acquisition was performed with a dwell time of 50 ms and Q1/Q3 set in unit resolution. At least three Q1/Q3 transitions were monitored for each tryptic peptide (see supplementary Table S1). The MRM transitions were identified experimentally from the nano LC-MS/MS IDA experiments; the transition of the S-palmitoylated peptide was calculated by adding the net mass of 238 Da.

Preparation of detergent-resistant membrane fractions- HEK293 cells stably expressing wild type A<sub>2A</sub>-receptor or A<sub>2A</sub>-receptor-R<sup>309C</sup> were grown in 150-mm culture dishes. Two dishes were used for each experiment. Membranes were subjected to density gradient centrifugation as described (28) with the following modifications: confluent cells were incubated in DMEM without FCS for 30 min, followed by addition of 3 μM CGS 21680 for further 30 min. Membranes were prepared as described above, resuspended in 0.5 mL buffer (50 mM Tris, pH 7.4, 2 mM EDTA, 150 mM NaCl) and homogenized by 10 aspiration cycles each, through a 20G and a 27G needle. Buffer (0.5 mL) containing 0.5% triton X-100 was added. Extraction was done on ice for 30 min. The lysate (0.7 mL) was adjusted to 42% (wt/wt) sucrose by adding 1.4 mL 59% sucrose in buffer, overlaid with 6 mL of 35% and 4 mL of 5% sucrose, and centrifuged at 217,000 g for 18 h. Samples were fractionated in 1 mL aliquots from the top (=fraction 1) to the bottom.
Single particle tracking- Cells (2*10^4/well) were seeded onto PDL coated glass-coVERSrips (diameter 25 mm) in 6-well culture dishes and maintained in medium (DMEM containing 25 mM glucose and 10% FCS) overnight at 37°C and 5% CO₂. A master mix was prepared, containing 3% casein (for blockage of non-specific binding sites), a 1:1 ratio of quantum dots (0.5 µg/µl final) reagent and anti-FLAG antibody (0.5 µg/µl final) in Krebs-HEPES buffer (10 mM HEPES.NaOH, pH7.5, 120 mM NaCl, 3 mM KCl, 2 mM MgCl₂, 2 mM CaCl₂, 22 mM glucose) and incubated for 15 min at 22°C. This was diluted to concentrations that allowed for binding of only few quantum dots per cell (usually in the range of 1:10^5). Coverslips were provided by Daniel Choquet (Univ. Bordeaux, France). Analysis was done as described (29). In addition, to allow for tracking of multiple particles, track.m-file was used for connection of trajectories (Daniel Blair and Eric Dufresne, http://physics.georgetown.edu/matlab/). At every step, a visual output was generated to verify the location of the imaged quantum dot and the connected trajectories. Mean squared displacement was calculated for each trajectory using the equation 1:

\[
MSD(n) = 1/(N-1-n) \sum [x(j+\Delta t) - x(jt)]^2 + [y(j+\Delta t) - y(jt)]^2 \quad (29=31).
\]

Diffusion coefficients (D_{1-4}) were estimated from a linear regression of the initial values of the mean square displacement (MSD) curve to the affine function (equation 2):

\[
MSD_{initial} (\Delta t) = 4*D_{initial}*(\Delta t + 2\sigma^2) \quad (32).
\]

The diffusion mode of the individual trajectories – i.e., random walk, transported or confined – was examined and analyzed as follows. Confined diffusion was separated from random walk by a predefined threshold: the pooled and averaged macroscopic diffusion coefficient (D_{mac}, obtained from fitting the first quarter of the MSD curve to the above equation) was calculated for each condition (basal, agonist-treated) and multiplied by (the one-tailed p-value of) 0.05 to define candidate outliers. Trajectories falling below this threshold were then fitted with MATLAB nonlinear least square regression employing the Trust-Region method to the equation for circular corrals (equation B14 in the appendix to ref. 34), where the factors: F₁ and F₂ are calculated (from the roots of Bessel function derivatives), while the AREA is estimated from the fit. The resulting apparent confinement radius (\rho_{app} = \sqrt{AREA/\pi}) was included for correlations with R² greater than 0.75, and a D_{mac}/D_{1-4} -ratio < 0.5, while all other potential confinements where discarded (because the brief, the region-based segmentation algorithm for detection was supplemented by a maximum likelihood estimation of a two-dimensional Gaussian mask, which accounts for the Poisson distribution of the photon count per pixel (30).
stochastic diffusion process may mimic confinement).

Statistics- Concentration-response curves, saturation curves and radioligand displacement curves were subjected to non-linear least squares curve fitting to equations describing a rectangular hyperbola, the sum of two hyperbolas or the Hill equation using a Marquardt-Levenberg algorithm. The improved fit associated with the choice of the more complex model was evaluated by an F-test based on the extra sum-of-squares principle. Diffusion coefficients were rounded to $10^{-3}$ $\mu$m$^2$/s and logarithmically transformed for further analysis. The cumulative frequency distributions of diffusivity (depicted as empirical cumulative distribution functions, CDFs) were analyzed using Kolmogorov-Smirnov-Test for two empirical distributions. The Kruskal-Wallis test followed by Dunn’s multiple comparisons were used to compare diffusivity of all four groups.

Results

Introducing a putative palmitoylation site does not affect cell surface expression and ligand binding of the $A_2\alpha$ receptor- The vast majority of group I (rhodopsin-like) G protein coupled receptors carries one (or two) palmitoylated cysteine(s) within the proximal portion of their C-terminus (typically some 20 residues away from the end of the seventh transmembrane segment (TM7)). Strikingly, the $A_2\alpha$-receptor does not have any cysteine residue in the proximal segment. Moreover, there is only a single cysteine in position 394 in the human receptor, which is absent in other species, e.g., mouse or rat. Finally, it was shown that the human $A_1$-receptor carries a palmitate thioester at a cysteine corresponding to position 309 of the $A_2\alpha$-receptor (35). This cysteine is conserved in the human $A_2\beta$- and $A_3$-receptors (Fig. 1A). We therefore substituted the arginine309 with cysteine and tested, if this renders the mutated receptor susceptible to palmitoylation by using two independent approaches, namely metabolic labeling (Fig. 1B) and mass spectrometry (Fig. 1C). HEK293 cells were transfected with plasmids encoding either wild type, mutated $A_2\alpha$-receptor or the $A_1$-receptor (as a positive control) with a FLAG-epitope at the N-terminus and a YFP fused to the C-terminus to visualize receptor expression (see below Fig. 2). Cells were metabolically labeled with $[^3H]palmitate and the receptors were immunopurified from detergent lysates. As shown in Fig. 1B, immunoprecipitation of both, the $A_1$-receptor (lane IP A1R) and the $A_2\alpha$-receptor-R$^{309}$C mutant (lane IP MT) recovered radioactively labeled bands migrating with an apparent molecular mass of approximately 70 kDa (marked with an arrow in Fig. 1B). The apparent molecular mass is consistent with that expected from the sum of the receptor and the fluorescent protein. The double bands arise from the differently glycosylated forms (36). For both receptor types, aggregates were seen at the top of the gel (marked with an asterisk in Fig. 1B). In contrast and as expected, we did not recover any labeled band in immunoprecipitates of the wild type $A_2\alpha$-receptor consistent with the fact that this receptor is not palmitoylated (lane IP WT in Fig. 1B).

In agreement with this observation the palmitoylation of C$^{309}$ of mutant $A_2\alpha$-receptor was verified by mass spectrometry. FLAG-tagged receptors (mutant and wild type) were immunopurified, digested with trypsin, the resulting peptides resolved by nano LC and analyzed by tandem MS. Using this approach we identified several receptor-specific peptides with a significant ProtScore (> 2) and a total receptor sequence coverage of 41%. The following two peptides were used for subsequent MRM experiments to identify the thioester-linked palmitate: SHVL*C*QQEPFK and QMESQPLPGER. Fig. 1C shows the extracted ion chromatogram from the MRM experiments and indicates the presence of the palmitate moiety in the mutant $A_2\alpha$-receptor-R$^{309}$C.

We also verified that the mutation of arginine at position 309 to cysteine did not affect folding and surface localization by visualizing the receptor (Fig. 2A and B) and by monitoring ligand binding (Fig. 2C and D). Both, the wild type (Fig. 2A) and the mutated $A_2\alpha$-receptor (Fig. 2B) were predominantly localized at the plasma membrane of stably transfected HEK293 cells. Similarly, binding of the $A_2\alpha$-receptor antagonist $[^3H]ZM241385 resulted in comparable saturation curves (Fig. 2C and D) with $K_D$ values of 3.3 ± 0.5 nM and 3.6 ± 1.0 nM for wild type $A_2\alpha$-receptor and the R$^{309}$C mutant, respectively. In the cell clones selected, $B_{max}$ values were similar with 1.4 ± 0.07 pmol/mg and 1.6 ± 0.16 pmol/mg for wild type $A_2\alpha$-receptor and $A_2\alpha$-receptor-R$^{309}$C, respectively.

Accumulation of cAMP in HEK293 cells expressing wild type and mutated $A_2\alpha$-receptor-GPCRs, such as $\beta$-adrenergic receptors or rhodopsin engage their cognate G protein by...
collision coupling. In contrast, it has long been known for the A2A-receptor that the rates of adenylyl cyclase activation via this receptor are incompatible with this model (21,22). Early studies rather revealed that the A2A-receptor was tightly coupled to the effector adenylyl cyclase in a manner that was accounted for by a restricted collision coupling model (20-22). Restricted collision coupling arises from the fact that the receptor can only activate a fraction of the cellular complement of its cognate G protein(s). In the case of the A2A-receptor, only a fraction of all available Gs is activated. This translates into a concentration-response curve in which increased receptor expression only enhances Emax (the maximum level of cAMP accumulation) but does not shift the EC50 to the left. Mechanistically, restricted collision coupling can be accounted for by a model where the cell surface membrane is compartmentalized and the receptor can only visit some of these compartments. We surmised that restricted collision coupling and the lack of palmitoylation are causally related. In order to test this hypothesis, several stable cell clones were obtained by selection with geneticin: three and four clones were selected for wild type and R309C mutant receptor, respectively, based on the following criteria: (i) the level of expression was reasonably similar for wild type receptor and the R309C mutated form; (ii) the concentration of receptor in the membrane covered a physiologically relevant range; (iii) clones with high (i.e., > 2 pmol/mg), expression levels were not included in the analysis. We determined cAMP accumulation after stimulation the cells with increasing concentrations of the A2A-selective agonist CGS21680. As anticipated, increasing expression levels of the wild type receptor only resulted in an increase in maximum accumulation of cAMP, but the EC50 of the agonist was in the range of 70 to 80 nM irrespective of the receptor levels (Fig. 3A and C). In contrast, increasing the expression levels of the A2A-receptor-R309C mutant resulted in a shift in the concentration response curve to the left (Fig. 3B). Accordingly, the EC50 was inversely related to the expression level of the mutant receptor (Fig. 3D). As a control, we replaced arginine in position 309 by alanine: the resulting mutant A2A-receptor-R309A also accumulated at the cell surface of HEK293 cells and effectively activated cAMP accumulation. In fact, the apparent agonist affinity was higher than in the wild type receptor (data not shown); however, EC50 values did not differ if cells expressing low and high levels of receptors were compared (2±0.2 and 2.1±0.3 nM, respectively). In addition, we examined the association of the agonist-activated wild type A2A-receptor and the mutated A2A-receptor-R309C with detergent-resistant membranes. Fig. 3E shows that in these sucrose gradients a large portion of the agonist-activated wild type receptors was retrieved in the flotillin-1-containing fractions. In contrast, the bulk of the agonist-activated A2A-receptor-R309C was recovered in the bottom fractions that were depleted of the raft marker flotillin-1.

Ternary complex formation by the A2A-receptor-R309C mutant- The substitution of arginine309 by a cysteine residue and the resulting palmitoylation did not affect antagonist affinity (Fig. 1C and D). However, it is evident from Fig. 3C and D that the EC50 of the agonist was increased at low expression levels of A2A-receptor-R309C when compared to the EC50 for the wild type receptor at corresponding expression levels. At high levels of expression, the difference in EC50 was eliminated and EC50 values were virtually identical. A trivial explanation may be a drop in agonist affinity induced by the mutation. We therefore determined the affinity of the agonist CGS21680 in competition experiments with the radiolabeled antagonist (Fig. 4). In membrane preparations, the A2A-receptor is known to form high-affinity agonist binding sites (reflecting the ternary complex HRG of agonist H, receptor and G protein) that are resistant to dissociation by guanine nucleotides. The ternary complex can, however, be disrupted by the addition of NaCl (17,37). This phenomenon is exemplified in Fig. 4A for the wild type A2A-receptor. The addition of NaCl converted the mixture of high- and low-affinity binding sites (ratio 3:1, Fig. 4A) into a homogeneous population of low-affinity binding sites. In contrast, addition GTPγS did per se not suffice to shift the competition curve (full symbols in Fig. 4A). Introducing a palmitoylation site did not affect the ability of the resulting A2A-receptor-R309C to form GTPγS-resistant high-affinity sites, but the proportion of high-affinity sites was reduced (Fig. 4B). By subjecting the data to curvilinear regression, the ratio of high- to low-affinity sites was estimated to be about 1:1. In contrast, the affinity of the agonist for the high-affinity sites was comparable to that seen for the wild type receptor (Kd = 0.14 ± 0.02 µM and 0.18 ± 0.06 µM for wild type and mutated receptor, respectively). Upon addition of NaCl, the competition curve was shifted to the right...
Effect of mutation on signaling via MAPK pathway. In endothelial cells, the A2A-receptor stimulates ERK/MAP kinase in a manner independent of Gs (8). This can be recapitulated in HEK293 cells (36), where stimulation of ERK does not require signaling via any G protein (26) but is contingent on recruitment of ARNO (the exchange factor for ARF6) (39). Stimulation of ERK and activation of cAMP accumulation appears to occur in physically separated membrane compartments (17). Hence, we explored whether the presence of the palmitoylation site also affected the receptor-mediated stimulation of ERK by immunoblotting for dually phosphorylated (=active) ERK1 and ERK2. As previously described (see e.g., 17), the time course of ERK phosphorylation was biphasic and was comparable in cells expressing wild type and mutated receptor (data not shown).

In cells expressing the wild type A2A-receptor, the concentration-response curves for the agonist CGS21680 were independent of receptor levels (cf. panels A, C and E in Fig. 5) with EC50 values of 6.3 ± 3.4 nM and 9.2 ± 3.7 nM for low and high receptor levels, respectively. In contrast, in cells expressing the A2A-receptor-R309C, the agonist concentration-response curve was shifted to the left as receptor levels increased (cf. panels B, D and F in Fig. 5) with EC50 values of 25.6 ± 6.7 nM and 6.3 ± 3.4 nM, respectively.

Determination of receptor mobility by single particle tracking. Regardless of whether cAMP accumulation or ERK activation was examined, the agonist activated A2A-receptor-R309C differed from the wild type receptor because the apparent EC50 depended on receptor levels. In addition, the agonist-liganded wild type A2A-receptor was associated to a larger extent with detergent-resistant membranes than the mutated A2A-receptor-R309C. We surmised that these altered properties ought to be linked to a change in receptor diffusion. We initially attempted to verify changes in receptor mobility by resorting to fluorescence recovery after photobleaching (FRAP). This approach suggested that wild type and mutant receptor differed, but in paired experiments we observed either variations in the time constant of fluorescence recovery or in the mobile fraction (data not shown, see also below). Because fluorescence recovery after photobleaching measures the properties of the receptor ensemble, these two variables are not independent. Accordingly, we labeled the receptors with quantum dots to measure the mobility of individual molecules. Two types of trajectories were observed: (i) in some instances, a receptor molecule was locally confined and did not escape from this local confinement over the entire observation period, i.e., 20 s (Fig. 6A). (ii) More frequently, receptors moved randomly over the cell surface resulting in trajectories consistent with a random walk over the entire observation period (Fig. 6B).

We calculated the diffusion coefficient for each individual particle from a plot of mean square displacement (MSD) against time intervals (Δt) by limiting our analysis to the first 25% of the acquisition time (i.e., a minimum of 100 total frames). The random walk mode (blue in Fig. 6C and D) was associated with an essentially linear dependence of area with time. In contrast, fitting the trajectory of a locally confined receptor (red curve in Fig. 6C and E) allowed for extracting the radius of the apparent confinement (Fig. 6E). On average the radii of the confined areas were 254 ± 112 nm and 187 ± 50 nm for wild type and mutant A2A-receptor, respectively, in the absence of CGS21680. In the presence of agonist, the radii diminished but they were also comparable, i.e., 116 ± 38 nm and 114 ± 55 nm for wild type and mutant A2A-receptor, respectively. If all data were pooled and the average diffusivity was determined (i.e., the area covered over time), it transpired that the addition of agonist reduced the diffusion coefficient for both, wild type and mutant A2A-receptor (cf. dashed and full lines in Fig. 6F). However, the effect of the agonist was modest in the case of A2A-receptor-R309C (blue lines in Fig. 6F) and substantially more pronounced for the wild type receptor (red lines in Fig. 6F). The variation in diffusivity of individual receptors is more readily accessible, if plotted as cumulative frequency distributions (CFDs) (Fig. 7A and B). This analysis highlighted that, under basal conditions,
the diffusivity of wild type receptor differed to a small – albeit statistically significant – extent (median \( D_{1(4)} = 0.14 \, \mu m^2/s \) and 0.12 \( \mu m^2/s \) for wild type receptor and for \( A_{2A} \)-receptor-R309C, respectively; \( p < 0.01 \) by Kolmogorov-Smirnov test) (Fig. 7A). Agonist activation resulted in a pronounced shift in the cumulative distribution curve for the wild type receptor (red curve in Fig. 7B; median \( D_{1(4)} = 0.05 \, \mu m^2/s \)) and only had a modest effect on \( A_{2A} \)-receptor-R309C (blue curve in Fig. 7B; median \( D_{1(4)} = 0.10 \, \mu m^2/s \)), which was nevertheless significantly different from the curve obtained in the absence of CGS21680 (\( p < 0.01 \) by Kolmogorov-Smirnov test). Thus wild type and mutant receptor differed substantially in their agonist-induced mobility change. This is best appreciated from the histograms shown in Figs. 7C-E. Under basal conditions, a Gaussian fit allows to resolve two populations of receptors in both, the wild type (Fig. 7C) and the mutant receptor (Fig. 7E). In the presence of agonist, the proportion of slowly diffusing molecules (orange curve in Fig. 7D) increased substantially in the wild type receptor at the expense of the rapidly moving species (cf. green lines in Fig. 7C and D). In contrast, agonist activation virtually eliminated the slowly moving species of \( A_{2A} \)-receptor-R309C (cf. Fig. 7E and F) and resulted in a homogenous population of rapid diffusivity (full line in Fig. 7F). The dashed lines in Fig. 7F represent the fit to a distribution assuming the presence of two receptor populations. It is evident that the fit is not significantly improved. The changes in diffusivity were also evaluated by subjecting the data set to a statistical comparison using a Kruskal-Wallis test followed by Dunn’s multiple post hoc comparisons; this confirmed that all observed diffusivities differed in statistically significant way (supplementary Fig. S1A).

Agonist treatment also slowed diffusion of the \( A_{2A} \)-receptor when assessed in hippocampal neurons transiently expressing a FLAG-tagged \( A_{2A} \)-receptor; the median diffusion rates were 0.56 \( \, \mu m^2/s \) and 0.22 \( \mu m^2/s \) in the absence and presence of agonist (n >250 trajectories in each condition, \( p<0.01 \)).

We also extracted the diffusion mode from the trajectories to determine the proportion of receptors subject to confinement defined by Saxton and Jacobson (33) (see Materials and Methods). In the inactive state, wild type and mutant receptor did not differ with 0.82% and 1.46%, respectively, of the molecules displaying highly restricted macroscopic mobility (Fig. 8A). Agonist activation did not affect confinement of the \( A_{2A} \)-receptor-R309C. In contrast, upon agonist activation, 19% of the wild type \( A_{2A} \)-receptors became immobile (Fig. 8B). This difference was statistically significant (\( p < 0.0001 \), Fisher’s exact test). We verified that the differences in mobility were related to the change in palmitoylation by pretreating cells expressing the wildtype \( A_{2A} \)-receptor and the \( A_{2A} \)-receptor-R309C with bromopalmitate, an inhibitor of palmitoylation. This pretreatment was predicted to eliminate the differences in mobility, i.e., the average diffusivity of agonist-activated \( A_{2A} \)-receptor-R309C ought to approach that of the wild type \( A_{2A} \)-receptor, if the difference was due to the presence and absence of the palmitoyl moiety. This was the case (Fig. 9A). The effect of bromopalmitate is most readily evident, if the cumulative frequency distribution is examined (Fig. 9B): the cumulative distribution curve of the agonist-activated mutant receptor approached that observed for the stimulated wild type receptor (cf. also Fig. 9B and Fig. 7B). In fact, in spite of an adequate power (with >440 recordings under each condition), we failed to detect any statistically significant differences in the distributions.

**Discussion**

In an isotropic membrane, a receptor is predicted to move freely and to engage its cognate G proteins by random collision. Thus, restricted collision coupling requires anisotropy: there must be areas of the membranes that cannot readily be visited by the activated receptor resulting in segregation of the receptor from the total available pool of its cognate G protein. We show here that, for the \( A_{2A} \)-receptor, this is indeed the case. The wild type \( A_{2A} \)-Receptor controls at last two signaling cascades (11), i.e., the \( G_z \)-dependent activation of adenylyl cyclase and the less well defined (\( G_x \)-independent) stimulation of ERK1/2, which relies on recruitment of ARNO to the C-terminus of the receptor (39) and on activation of RAS (8,38). For both, agonist-induced cAMP accumulation and ERK-phosphorylation, the \( EC_{50} \) was independent of the expression level of the \( A_{2A} \)-receptor. This hallmark of restricted collision coupling was eliminated by introducing the palmitoylation site into the C-terminus of the \( A_{2A} \)-receptor irrespective of which pathway was examined. In addition, palmitoylation had a pronounced effect on receptor mobility, in particular in the agonist-bound state. The following arguments support the conclusion that the switch from restricted collision coupling to
unrestricted collision coupling are the result of the observed changes in diffusion mode and in diffusibility: (i) restricted collision coupling is contingent on confinement of the receptor. We observed a large increase in confinement upon agonist binding to the wild type receptor. This, however, was not seen if a palmitoylation site was engineered into the C-terminus. We stress that our definition of confinement was conservative by focusing only those trajectories that were adequately described by equation 3. Temporary confinements were not considered. (ii) In addition, in the presence we observed a pronounced redistribution of wild type A2A-receptors from the fast mobile pool to a slowly diffusing population. This redistribution was absent in the palmitoylated pool. By definition, in a slowly diffusing receptor species, the number of productive encounters with its cognate G protein (or any other signaling moiety) must be lower than in a rapidly moving receptor.

The number of high-affinity ternary complexes observed with the palmitoylated version of the A2A-receptor was modestly reduced. This can be rationalized by assuming that, because of its enhanced mobility, the palmitoylated version of the A2A-receptor is more likely to be separated from Gs in broken cell preparations. Accordingly, a physical separation of receptors from G proteins is more probable and the likelihood increases that the receptor will be trapped in a vesicle that does not contain any Gs. Alternative explanations, i.e., a reduced affinity of the receptor for the agonist or the G protein, cannot account for the decrease in ternary complexes formed by the palmitoylated version of A2A-receptor: in the uncoupled state, we observed a modestly increased affinity of the palmitoylated receptor for the agonist. This can be explained by assuming that the agonist-liganded structure is stabilized by membrane anchoring of helix 8 via the palmitoyl-moiety. Alternatively, there may be an effect that results from the loss of the positive charge provided by the side chain of arginine 309. Similar to the wild type receptor, the palmitoylated receptor formed a tight GTPγS-resistant complex. In this ternary complex, agonist affinity was comparable in the wild type and palmitoylated receptor. We therefore conclude that the presence or absence of palmitoylation does not affect G protein-coupling. Several earlier observations support this conclusion. Mutation of the palmitoylated cysteine to alanine does not affect G protein-coupling of the A1-adenosine receptor (35). This is consistent with the observation that the human A1-adenosine expressed in E.coli, which does not support palmitoylation, interacts with G protein with the same affinity as the receptor in brain membranes (40). We note that in several instances palmitoylation has been reported to be essential for G protein coupling; however, because these assays are based on indirect readouts of G protein-coupling in cells, they are confounded by additional events, e.g., enhanced desensitization of the depalmitoylated receptor (reviewed in ref. 41). In fact, all GPCRs that have been expressed in E.coli and reconstituted in their depalmitoylated state with G proteins bind their cognate G proteins with high affinity and with the appropriate selectivity (40,42,43). Earlier work also suggested a rapid agonist-induced turnover of the palmitoyl-moiety in the C-terminus of the β2-adrenergic receptor, the paradigmatic G protein-coupled receptor (44). It is worth noting though that these and related experiments were done in SF9 cells and that they pointed to a link between depalmitoylation and phosphorylation (45). However, when examined in mammalian cell lines and quantified by mass spectrometry, palmitoylation of the β2-adrenergic receptor was remarkably stable: there were no appreciable changes in palmitoylation up to 30 min after agonist treatment (46). After pretreatment with bromopalmitate, an inhibitor of palmitoylation, we observed a pronounced change in the diffusion of the agonist-activated A2A-receptor-R309C but not of the wild type receptor. These observations provide circumstantial evidence to support the assumption that agonist-treatment does not result in a marked change in the palmitoylation state of the mutated receptor.

Our experiments were designed to assess the diffusion mode and the change in diffusivity upon agonist-activation of a G protein-coupled receptor by using single molecule tracking. We are not aware of any other investigation that has directly visualized the local confinement of an agonist-activated G protein-coupled receptor. Accordingly, there is only a limited data set available for comparison. Using fluorescence recovery after photobleaching (FRAP), we previously failed to detect any change in A2A-receptor diffusion upon agonist binding (17). FRAP also failed to detect consistent changes, when we compared the wild type A2A-receptor and the palmitoylated variant: in some instances, paired experiments revealed a difference in the
recovery time constant, in the mobile fraction or both. This variability can be attributed to the ensemble nature of the FRAP recordings, which average the large heterogeneity of receptors detected by single particle tracking. Accordingly, transient confinement events may affect recovery time constant and/or the mobile fraction depending on the relative distribution of receptors subjected to synchronized bleaching in a given membrane strip. In fact, if we arithmetically average diffusion coefficients without considering the underlying distribution, we observe a considerable overlap in the mean diffusion coefficients (supplementary Fig. S1B). This suggests that FRAP recordings may only be sensitive enough to detect agonist-induced changes in mobility, if the agonist-induced conformational change translates into a mobility shift in the dominant population of the diffusing particles. In contrast, methods that track the mobility of individual particles provide information on the heterogeneity of the molecules.

The presence of an agonist reduced the mobility of both, the wild type A2A-receptor and to a less pronounced extent also that of the palmitoylated version. The underlying cause is not clear. Stimulation by the agonist ought to promote the formation of a receptor-G protein complex and possibly larger complexes (termed “signalosomes”) that result from the association with one or several effectors. Intuitively, this situation is expected to slow receptor movement. However, agonist binding does not uniformly slow receptor diffusion: accelerated diffusion was observed for the (G _i/G _o-coupled) 5-HT _1A receptor (47), but the mobility of the (G _i/G _o-coupled) D _2-receptor was slowed by agonist occupancy (17). Similarly, analysis of single particles by fluorescence correlation spectroscopy showed that the diffusivity of the (G _i-coupled) CRF1-receptor was enhanced upon agonist binding while that of the related (G _i-coupled) CRF2-receptor was not affected (48). This suggests that the agonist-induced change in diffusivity is an intrinsic property of the receptor protein. CRFR1 and CRFR2 differ mainly in their hydrophobic core, i.e., in that region of the receptor that is subject to the viscous drag of the lipid bilayer. Similarily, the palmitoylated A2A-receptor-R309C diffused more rapidly in both, the basal, unliganded and the agonist-activated state than the wild type receptor. In fact, in the presence of agonist, the palmitoyl-modification rendered the distribution more homogeneous by eliminating, both the slow portion of the receptor pool and the highly mobile tail of the fast population. In the X-ray crystal structure, the shape of the agonist-liganded receptor does not differ dramatically from that of the antagonist-bound receptor (13-15). However, it is worth pointing out that these structures lack a crucial component, i.e., the G protein: the C-terminus of the G protein α-subunit has to be accommodated in a cavity created on the intracellular side (49). The resulting change in the shape of the receptor is likely to create a hydrophobic mismatch that may augment the viscous drag. In fact, based on molecular dynamics simulations, it has been argued that in the unliganded state the ligand binding pocket of the A2A-receptor may be invaded by phospholipid headgroups that pry apart transmembrane helix 1 and helix 2; cholesterol eliminates the hydrophobic mismatch and stabilizes the structure (16). It is conceivable that the presence of the palmitoyl moiety also exerts a stabilizing effect on the helices and thus obviates the requirement for a cholesterol rich environment. This speculative interpretation is also consistent with the observation that the agonist-activated wild type A2A-receptor was more likely to visit confined areas than the version of the receptor engineered to be palmitoylated. The calculated size of the confinement area is consistent with the size of a hypothetical lipid raft. The absence of the palmitoylated A2A-receptor-R309C in confined areas is counterintuitive, for palmitoylation is generally thought to promote confinement in lipid rafts (41). However, a systematic proteomic survey of raft and non-raft membranes shows that S-palmitoylation does not represent an obligatory signal for raft association (50). In fact, there is at least one example, where palmitoylation prevents a protein (i.e., the isoform-1 of the endothelial membrane protein TEM8) from entering lipid rafts, while mutation of the acceptor cysteines removes this constraint and allows for entry into rafts (51). Thus, the most parsimonious explanation of restricted restricted collision coupling is to attribute it to a local confinement of the A2A-receptor in a structure corresponding to a (putative) lipid raft.

References


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Footnotes
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‡These two authors contributed equally.

Abbreviations
The abbreviations used are: ARNO, ADP-ribosylation factor nucleotide-binding site opener (a guanine nucleotide exchange factor for ARF6); CGS21680, 3-[4-[2-[6-amino-9-((2R,3R,4S,5S)-5-(ethylcarbamoyl)-3,4-dihydroxy-oxolan-2-yl]purin-2-yl]amino]ethyl]phenyl]propanoic acid; ERK1/2, extracellular signal-regulated kinase-1 & -2 = MAPK, p44/p42 mitogen-activated protein kinase; FRAP, fluorescence recovery after photobleaching; IDA, information-dependent acquisition; LC, liquid chromatography; MRM, multiple reaction monitoring; MS, mass spectrometry; MSD, mean square displacement; XAC, xanthine amino congener; YFP, yellow fluorescent protein; ZM241385, 4-(2-[7-amino-2-(2-furyl)-[1,2,4]-triazolo-[2,3-a]-[1,3,5]-triazin-5-ylamino]ethyl)phenol
Figure legends

Fig. 1. Comparison of the C-terminal sequences of the four human adenosine receptors (A) and palmitoylation of the A2A-receptor-R309C detected by autoradiography (B) and by mass spectrometry (C). A: The sequences of the carboxyl termini of the four human adenosine receptors are shown starting with the first residue after the seventh transmembrane helix. The cysteine residue that is the (putative) palmitoylation site is underlined; it is present in the A1-, A3-, and A2B-receptors but not in the A2A-receptor, which has an arginine residue (R309) in this position. It is also evident that the C-terminus of the A2A-receptor is substantially longer than that of any of the other receptors. B: HEK293 cells (3*10^5 cells) were transiently transfected with plasmids encoding the YFP-tagged wild type A2A-receptor (lanes labeled WT), A1-receptor (A1R) or A2A-receptor-R309C mutant (MT) and incubated in the presence of [3H]palmitate for 1 h. Thereafter, the cells were lysed and solubilized material was immunoprecipitated with an antibody against GFP, lysates (LYS; 20 µg), supernatants (SNT, 20 µg) and immunoprecipitates (IP, corresponding to 2.5*10^5 cells) were electrophoretically resolved and radioactively labeled bands were visualized by fluorography. The arrow indicates the position of the palmitoylated A2A-receptor-R309C band and the asterisk aggregated material. (C) Mass spectrometry. Extracted ion chromatogram from the multiple reaction monitoring (MRM) experiments to identify the presence of palmitoylation at the C-terminus of the A2A-receptor-R309C. Three specific MRM Q1/Q3 transitions of the mutant tryptic peptide (“SHVLC*QQEPFK”) and the A2A-receptor internal control peptide (“QMESQPLPGER”) were monitored as outlined under Materials and Methods and are indicated by dashed lines. The chromatogram was prepared with the Analyst 1.5.2 software using Gaussian smoothing and background subtraction.

Fig. 2. Expression of wild type A2A-receptor and A2A-receptor-R309C in HEK293 cells. Representative example of YFP-tagged wild type A2A-receptor (A) and A2A-receptor-R309C (B) visualized by confocal microscopy. The bar represents 10 µm. C and D: Binding of the radioligand antagonist [3H]ZM241385 to membranes prepared from HEK293 cells stably expressing wild type A2A-receptor (C) and A2A-receptor-R309C (D). The membranes (5 µg/assay) were incubated with the indicated concentrations of [3H]ZM241385 as outlined under Materials and Methods. Non-specific binding was determined in the presence of 10 µM XAC and was subtracted. Non-specific binding was less than 10% in the K_D-concentration range. Data are means from duplicate determinations in a representative experiment that was reproduced three times with similar results.

Fig. 3. Accumulation of [3H]cAMP in HEK293 cells stably expressing wild type A2A-receptor (A,C) and A2A-receptor-R309C (B,D) at different levels and association of agonist-activated receptors with detergent-resistant membranes (E). HEK293 cells were stably transfected with plasmids encoding wild type A2A-receptor and A2A-receptor-R309C and clones (wt #7, #10, #6; R309C #8, #13,#18,#14) selected. Receptor expression levels were determined in binding experiments as in Fig. 2 and the corresponding B_max values are plotted in panels B and C. Cells (3*10^5/well) were metabolically labeled with [3H]adenine for 16 h. After addition of fresh medium, cAMP production was stimulated by the indicated concentrations of CGS21680 for 20 min. Data are means ± S.D. from three experiments. The red line in A and B connects the EC50 values in the individual curves. In panels C and D, EC50 values calculated from individual concentration-response curves are plotted as a function receptor levels (B max) to illustrate the inverse correlation in cells expressing A2A-receptor-R309C (D) and the absence of any correlation in cells expressing the wild type receptor (C). E: HEK cells expressing either YFP-tagged wild type A2A-receptor or A2A-receptor-R309C were stimulated with 3 µM CGS21680 for 30 min. Membranes were prepared from these cells, extracted with 0.25% triton X-100 and subjected to centrifugation on a discontinuous isocrose gradients (by flotation). After ultracentrifugation, fractions (1 mL) were collected from the top. All fractions were analyzed; shown are fractions 3, 4, 5, 6, 10 and 11 (25 µL each) and the loaded lysate (L; 6 µl) that were resolved on the same denaturing polyacrylamide gel, transferred to nitrocellulose, and immunoblotted using antibodies to A2A-receptor, flotillin-1 and Gβ. The light fractions from the top of the gradients are on the left, the heavy bottom fractions on the right. The position of fractions containing detergent-resistant membranes (DRMs) is indicated.

Fig. 4. Competition of the agonist CGS21680 for binding of the antagonist [3H]ZM241385 to membranes of HEK293 cells stably expressing wild type A2A-receptor (A) and A2A-receptor-
Membranes (30 - 40 µg) prepared from HEK293 cells (clones wt #6 and R309C #14, cf. Fig. 3) were incubated with 3 nM [3H]ZM241385 and the indicated concentrations of CGS21680 in the absence (open circle) or presence of 100 µM GTPγS (closed circle), 150 mM NaCl (open square) or 100 µM GTPγS and 150 mM NaCl (closed square). Data represent mean ± SEM of ≥ three independent experiments carried out in duplicates.

Fig. 5. Concentration-response curve for agonist-stimulated ERK phosphorylation in HEK293 cells stably expressing wild type A2A-receptor (A,C,E) and A2A-receptor-R309C (B,D,F). Confluent serum-starved cells (3*10⁵/well) expressing wild type A2A-receptor at low (A, clone wt #7, cf. Fig. 3) or high levels (C, clone wt #6) or A2A-receptor-R309C mutant at low (B, clone R309C #8) or high levels (D, clone R309C #14) were stimulated with the indicated nM concentrations of CGS21680 for 5 min. Stimulation by 1 µM phorbol myristate acetate (PMA) for 20 min served in panels A-D as a positive control and to allow for comparison between different blots. Aliquots of cellular lysates (20 µg) were applied to SDS-polyacrylamide gels. After electrophoretic resolution and transfer to nitrocellulose, the level of active ERK1/2 was assessed by immunoblotting with an antiserum recognizing the dually phosphorylated active enzyme (p-ERK), or an antiserum against holo-ERK1/2 (holo), (loading control). Concentration-response curves were plotted by averaging densitometric estimates from three independent experiments with low and high expressing wild type (E) and mutant receptor (F); error bars indicate SEM.
activated state is statistically significant (p < 0.0001, Fisher’s exact test), while this is not the case for the A2A-receptor-R309C.

Fig. 9. Averaged mean square displacements (MSD) (A) and cumulative frequency distributions (CDF) (B) of agonist-activated wild type A2A-receptor and of A2A-receptor-R309C in cells subjected to a pretreatment with 2-bromopalmitate. HEK293 cells stably expressing wild type A2A-receptor and A2A-receptor-R309C were pretreated with 100 µM 2-bromopalmitate (2BP, dashed lines) for 48 h. Control cells were maintained in medium (full lines). Thereafter cells were stimulated with 10 µM CGS21680; trajectories of quantum dot labeled receptors were recorded and averaged mean displacements (panel A) and cumulative frequency distributions (panel B) were calculated as outlined in the legends to Fig. 6 and Fig. 7, respectively. The cumulative frequency distributions are based on 445 (wt-A2A-receptor, red line) and 461 (A2A-receptor-R309C, blue line) initial D1-4 diffusion coefficients and they were compared by a Kolmogorov-Smirnov test, which did not reveal any statistically significant difference (p>0.05).
Fig. 1

A

A₁: RIQKFRVTFLKIWNDHFRCPAPPIDEDLPEERPDD
A₃: KIKKFKETYLLILKACVCHPSDLDTSIEKNS
A₂B: RNRDFRTFHKIIISRYLLCQADVKSNGQAGVQPALGVGL
A₂A: RIREFRQTRKIIIRSHVLRQQEPFKAAGTSARVALAHGSDGEQVSLRNLGHPGVWANGSAPHPERRPNGYALGLVSGGSAQESGNTGLPDVE7LSHELKGCPEPGLDDPLAQDGAGVS

B

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C

Retention time (min)

Intensity (cps)

“QMESQLPGER”

Q₁/Q₃ (Da)

638.41 ± 458.20
638.41 ± 668.40
638.41 ± 1012.50

“SHVLQQEPFK”

Q₁/Q₃ (Da)

777.80 ± 225.20
777.80 ± 324.40
777.80 ± 391.60
Fig. 2

A2A R-YFP wild type

A2A R-YFP R^{309}C

C

D


Fig. 3

A

B

C

D

E

wild type A2\(\text{A}\)R

A2\(\text{A}\)R-R\(^{309}\text{C}\)

[kDa] 75 - 48 - 35 -

DRM

DRM
wild type A$_{2A}$-receptor

[3H]ZM241385 bound [% of control]

GTP$_{\gamma}$S  
control  
GTP$_{\gamma}$S + NaCl  
NaCl

CGS21680 [µM]

A

A$_{2A}$-receptor R309C

[3H]ZM241385 bound [% of control]

GTP$_{\gamma}$S  
control  
GTP$_{\gamma}$S + NaCl  
NaCl

CGS21680 [µM]

B
Fig. 6

A

B

C

D

E

F

R309C+CGS21680
Random Walk

WT+CGS21680
Conf Radius=0.1 μm
Fig. 7

(A) Cumulative distribution of diffusion coefficients for WT and R299C mutants. 
(B) Cumulative distribution of diffusion coefficients for WT-A2A and R299C-A2A mutants.

(C) Histogram of relative frequency of diffusion coefficients for WT. 
(D) Histogram of relative frequency of diffusion coefficients for WT-A2A.

(E) Histogram of relative frequency of diffusion coefficients for R299C. 
(F) Histogram of relative frequency of diffusion coefficients for R299C-A2A.
Fig. 8

**A**

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**B**

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Fig. 9

A

B

WT-42AR + CGS21680
R309C-42AR + CGS21680
WT-42AR + CGS21680 + 2BP
R309C-42AR + CGS21680 + 2BP

cumulative frequency

<NSD> [µm²]

Δt [s]

D_{t-d} [µm²/s]
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<td>777,80</td>
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Q1: molecular weight (MW) of MRM precursor ion; Q3: MW of fragment ions after dissociation; CE: collision energy; *palmitoylation of cysteine
Supplementary Fig. 1. **Comparison of diffusivity of the wild type A<sub>2A</sub>-receptor and A<sub>2A</sub>-receptor-R<sup>309C</sup> in the absence and presence of the agonist CGS21680 (10 µM).** A: The median diffusivity ($D_{1-4}$) was calculated from the data shown in Fig. 7. The boxed area covers the 25 – 75 interquartile range. The whiskers span 4 interquartile ranges to define outliers, which are marked as crosses. A Kruskal-Wallis test followed by Dunn’s multiple comparisons indicates that all data sets differ in a statistically significant manner (p<0.01). B: Arithmetic means ± SD are shown to illustrate the fact that ensemble-based averaging (such as fluorescence recovery after photobleaching/FRAP) may fail to detect differences in receptor diffusion. Please check!
Supplementary Fig. 1. **Comparison of diffusivity of the wild type A$_{2A}$-receptor and A$_{2A}$-receptor-R$^{309}$C in the absence and presence of the agonist CGS21680 (10 µM).** *A*: The median diffusivity ($D_{1-4}$) was calculated from the data shown in Fig. 7. The boxed area covers the 25 – 75 interquartile range. The whiskers span 4 interquartile ranges to define outliers, which are marked as crosses. A Kruskal-Wallis test followed by Dunn’s multiple comparisons indicates that all data sets differ in a statistically significant manner ($p<0.01$). *B*: Arithmetic means ± SD are shown to illustrate the fact that ensemble-based averaging (such as fluorescence recovery after photobleaching/FRAP) may fail to detect differences in receptor diffusion.

Supplemental Videos 1&2: AVI-Videos were generated in MATLAB® R2009a (The MathWorks™) from an overlay of the recorded bright field image (16 bit grayscale TIFF image converted to truecolor 8 bit RGB image) with an artificially colored dot based on the intensity- and positional- information of the low-pass filtered quantum dot from the original stack-file. Apparent fluctuations in size are due to blinking related changes in fluorescence intensity, while a small black halo was added for contrast.

1.) “random_walk”: shows an A$_{2A}$-receptor-R$^{309}$C mutant under CGS21680 agonist treatment, over 1000 frames of 86 pixels by 80 pixels @ 50 frames per second, (pseudo)blue colored QDot.

2.) "confined_walk": shows a wild type A$_{2A}$-receptor under CGS21680 agonist treatment over 1000 frames of 96 pixels by 92 pixels @ 50 frames per second, (pseudo) red colored QDot.
Reengineering the collision coupling and diffusion mode of the A2A-adenosine receptor: palmitoylation in helix 8 relieves confinement
Simon Keuerleber, Patrick Thurner, Christian W. Gruber, Juergen Zezula and Michael Freissmuth

J. Biol. Chem. published online October 15, 2012

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