Restricted Collision Coupling of the A<sub>2A</sub> Receptor Revisited

EVIDENCE FOR PHYSICAL SEPARATION OF TWO SIGNALING CASCADES*

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The A<sub>2A</sub>-adenosine receptor is a prototypical G<sub>i</sub> protein-coupled receptor but stimulates MAPK/ERK in a G<sub>i</sub>-independent way. The A<sub>2A</sub> receptor has long been known to undergo restricted collision coupling with G<sub>i</sub>; the mechanistic basis for this mode of coupling has remained elusive. Here we visualized agonist-induced changes in mobility of the yellow fluorescent protein-tagged receptor by fluorescence recovery after photobleaching microscopy. Stimulation with a specific A<sub>2A</sub> receptor agonist did not affect receptor mobility. In contrast, stimulation with dopamine decreased the mobility of the D<sub>2</sub> receptor. When coexpressed in the same cell, the A<sub>2A</sub> receptor precluded the agonist-induced change in D<sub>2</sub> receptor mobility. Thus, the A<sub>2A</sub> receptor did not only undergo restricted collision coupling, but it also restricted the mobility of the D<sub>2</sub> receptor. Restricted mobility was not due to tethering to the actin cytoskeleton but was, in part, related to the cholesterol content of the membrane. Depletion of cholesterol increased receptor mobility but blunted activation of adenylyl cyclase, which was accounted for by impaired formation of the ternary complex of agonist, receptor, and G<sub>protein</sub>. These observations support the conclusion that the A<sub>2A</sub> receptor engages G<sub>i</sub> and thus signals to adenylyl cyclase in cholesterol-rich domains of the membrane. In contrast, stimulation of MAPK by the A<sub>2A</sub> receptor was not impaired. These findings are consistent with a model where the recruitment of these two pathways occurs in physically segregated membrane microdomains. Thus, the A<sub>2A</sub> receptor is the first example of a G protein-coupled receptor documented to select signaling pathways in a manner dependent on the lipid microenvironment of the membrane.

In the fluid mosaic model, the lipid bilayer is an isotropic milieu, in which membrane-embedded proteins diffuse in two dimensions and thus collide at random with each other (1). When applied to G protein-coupled receptors, the model predicts that G protein-coupled receptors move in a random walk, and, upon activation, this allows them to engage their cognate G proteins. This “collision coupling” mode of activation was validated in studies using the β-adrenergic receptor and its coupling to the effector enzyme adenylyl cyclase in turkey erythrocytes (2). However, experiments with the A<sub>2A</sub>-adenosine receptor in turkey erythrocytes revealed kinetic properties of adenylyl cyclase activation that were incompatible with the collision coupling model. The results indicated a tight coupling of the adenosine receptor to G<sub>i</sub>, (3, 4); the term “restricted collision coupling” was coined to account for this altered mode of coupling. Restricted collision is not a feature unique to the avian A<sub>2A</sub>-adenosine receptor, because it was also documented for the human A<sub>2A</sub> receptor in platelet membranes (5). In addition, the A<sub>2A</sub>-adenosine receptor has the unusual feature of forming a tight complex with G<sub>i</sub>, which persists in detergent solution, which is resistant to guanine nucleotides and which requires the combined addition of guanine nucleotides and high concentrations of NaCl to destabilize the ternary complex (6, 7). This argues for “precoupling” (i.e. a preexisting complex between receptor and G protein, which is already formed in the absence of agonist). The combination of pre-coupling and a tight association, which is not dissociated in the presence of guanine nucleotides, by definition, gives rise to activation kinetics of adenylyl cyclase indistinguishable from restricted collision coupling.

The mechanistic basis for restricted collision coupling has remained elusive. Here, we explored two alternative working hypotheses; the restricted mobility of the receptor may either arise from its interaction with the cytoskeleton, specifically the cortical actin cytoskeleton, or be due the anisotropy of the cell membrane resulting from its cholesterol content. We reexamined the restricted collision coupling model by visualizing the mobility of the A<sub>2A</sub> receptor by using fluorescence recovery after acceptor photobleaching (FRAP).4 In the present work, we demonstrated that it is the lipid composition of the cell membrane that is important for restricting the mobility of the A<sub>2A</sub> receptor. Depletion of cholesterol specifically impaired coupling of the A<sub>2A</sub> receptor to G<sub>i</sub> and thus activation of adenylyl cyclase. In contrast, the A<sub>2A</sub> receptor still triggered (G<sub>i</sub>-independent) stimulation of MAPK.

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4 The abbreviations used are: FRAP, fluorescence recovery after photobleaching; ARNO, ADP-ribosylation factor nucleotide-binding site opener (a guanine nucleotide exchange factor for ARF6); CFP, cyan fluorescent protein; CRF, corticotropin-releasing factor; FRET, fluorescence energy resonance transfer; GPCR, G protein-coupled receptor; NFRET, net FRET; PKA, protein kinase A; YFP, yellow fluorescent protein; XAC, xanthine amino congener; Δ-PDMP, Δ-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; ANOVA, analysis of variance.
EXPERIMENTAL PROCEDURES

Materials and Reagents—[3H]Adenine was from PerkinElmer Life Sciences, and adenosine deaminase was from Roche Applied Science. CGS21680 and [3H]ZM241385 (specific activity 27.4 Ci/mmol) were from Tocris Cookson Ltd. (Bristol, UK). CRF was obtained from Polypeptide (Wolfenbuettel, Germany).

Materials required for SDS-PAGE were from Bio-Rad. Fetal calf serum was from PAA Laboratories (Linz, Austria); Dulbecco’s modified Eagle’s medium, nonessential amino acids, β-mercaptoethanol, gentamicin, G418 (geneticin), Lipofectamine, and Lipofectamine Plus reagent were obtained from Invitrogen. cAMP, filipin III, forskolin, l-glutamine, haloperidol, latrunculin A, methyl-β-cyclodextrin, quinpirole, pertussis toxin, sulpiride, streptomycin, Triton X-100, xanthine amino congener (XAC), anti-FLAG-M2-affinity gel, and peroxidase-conjugated anti-FLAG monoclonal antibody were purchased from Sigma, and d-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (d-PDMP) was from Cayman Chemicals (Ann Arbor, MI). Rabbit anti-green fluorescent protein living Colors A.v. peptide antibody was from Clontech. Horseradish peroxidase-conjugated anti-mouse and anti-rabbit immunoglobulin antibodies were from Amersham Biosciences. The immunoreactive bands on nitrocellulose blots from Whatman (Dassel, Germany) were detected using SuperSignal chemiluminescence substrate from Pierce or ECL plus from Amersham (Dassel, Germany). Scintillation fluid was Rotiscint Ecoplus (Carl-Roth GmbH, Karlsruhe, Germany).

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DNA Constructs—Plasmids encoding YFP-tagged catalytic and CFP-tagged regulatory subunit of PKA (8) were generous gift from Manuela Zaccolo (University of Padua, Italy); the plasmid driving expression of CFP-Rab 5 was kindly provided by Alexander Sorkin (University of Colorado, Denver, CO). The plasmid coding for the CFP-tagged human D2-dopamine receptor was generated as described for the YFP-tagged version (9); the construction of the plasmids has been described, which drive the mammalian expression of the (N-terminally) FLAG- or (C-terminally) YFP-tagged full-length and C-terminally truncated versions of the A2A receptor (10). cDNA coding for mouse CRF2 receptor was kindly provided by W. Vale (Clayton Foundation Laboratories for Peptide Biology, La Jolla, CA).

Cell Culture, Radioligand Binding Assay, MAPK Stimulation, and Cellular cAMP Accumulation—HEK293 cells were maintained in Dulbecco’s modified Eagle’s medium, PC-12 cells were at 5% CO2, 95% air and 37 °C. Culture media were supplemented with 10% fetal calf serum, 2 mM l-glutamine, β-mercaptoethanol, and nonessential amino acids. Cells were transfected by using Lipofectamine Plus™. Rat striatal neurons were isolated on E17 and transfected with the PKA cDNA constructs using a rat neuron kit (Amaxa, Gaithersburg, MD) according to the manufacturer’s instructions. Briefly 5 × 10^6 cells were centrifuged at 500 × g and resuspended in 0.1 ml of nucleofector solution. After the addition of 2 µg of DNA, cells were electroporated with program O-003. Dulbecco’s modified Eagle’s medium plus 10% fetal calf serum (0.5 ml) was immediately added, and cells were allowed to recover for 5 min at 37 °C, subsequently seeded on poly-d-lysine-coated coverslips, and maintained in neurobasal modified Eagle’s medium supplemented with B27 and 1% penicillin/streptomycin. Fluorescence resonance energy transfer (FRET) measurements were performed after 5 days in vitro.

Binding of the antagonist [3H]ZM241385 was determined in a final volume of 0.5 ml of Dulbecco’s modified Eagle’s medium containing ~5 × 10^4 suspended cells and radioligand concentrations ranging from 0.1 to 15 nM. Nonspecific binding was determined in the presence of 5 µM XAC and was less than 10% in the Kd concentration range. The reaction mixture was kept in the cell culture incubator at 37 °C for 45 min. The reaction was terminated by filtration over glass fiber filters using a cell harvester. In competition experiments, the radioligand concentration was 2 nM. To disrupt ternary complexes, 100 µg/mL GTPγS was included in the reaction mixture, and cells were permeabilized by a freeze-thaw cycle (flash freezing in liquid nitrogen followed by rapid thawing).

The conditions for stimulation of MAPK are outlined in Ref. 11. Immunoreactive bands were visualized with antibodies directed against dually phosphorylated p42 (ERK2) and p44 (ERK1) MAPK or holo-ERK (as a loading control) and quantified using the densitometric quantification program Scion Image by Scion Corp. (Frederick, MD). The cellular ATP pool was metabolically prelabeled by incubating cells (10^5 cells/well of a 6-well dish) for 16 h with 0.2 µCi of [3H]adenine. Assay conditions for agonist-induced cAMP accumulation were as described in Ref. 12.

Fluorescence Microscopy, FRET, and FRAP—Cells were seeded on coverslips and (co-)transfected with plasmids encoding YFP-tagged A2A receptor (or truncated versions thereof) and CFP-tagged D2 receptor. Twenty-four hours after transfection, the coverslips were mounted in the microscope chamber. FRET microscopy was done as with a Zeiss Axiovert 200M inverted epifluorescence microscope equipped with a CoolSNAP fx cooled CCD camera (Photometrics, Roper Scientific, Tucson, AZ). For dual emission ratio imaging, we used an excitation filter at 436 nm, a dichroic mirror with a cut-off at 550 nm, and two emission filters (476 nm for CFP and 535 nm for YFP; Chroma Technology, Rockingham, VT) using an automated filter wheel (Ludl Electronic Products Ltd., Hawthorne, NY). An additional excitation filter at 500 nm for YFP was used for three-filter FRET microscopy (13). Fluorescence images were background-corrected and analyzed with MetaFluor software (Universal Imaging, West Chester, PA). Integration time was 200 ms/image. To measure donor recovery after acceptor photobleaching, we acquired a donor (CFP) image before and after photobleaching using the YFP setting for 90 s (excitation...
500 nm, dichroic mirror 525 nm, and emission 535 nm) to calculate a ratio image. Fluorescence images were analyzed using the MetaSeries software MetaFluor and MetaMorph (release 4.6, Universal Imaging Corp., Downington, PA). Photo-bleaching FRET microscopy was done by continuous illumination with a 100-W mercury lamp and the CFP filter set with time series imaging for 1 min (with the acquisition of one image every 2 s), which was sufficient to bleach the donor to an extent of less than 20% (14). Regions of interest were selected over the membrane, and fluorescence emission intensities were quantified using the NIH Image software. The resulting decay curves were fitted to the equation for a single exponential decay approaching a constant value: fluorescence intensity = \( A_0 \times e^{-kt} + b \), where \( A_0 \) denotes the starting value, \( b \) denotes the final fluorescence signal, and \( K \) is the decay constant. The time constant \( \tau \) (fluorescence lifetime) is defined as \( 1/K \). Confocal microscopy was performed under oil immersion using a laser scan microscope (Zeiss Axiovert LSM510). FRAP analysis was carried out in cells transfected with YFP-tagged \( \text{A}_{2\alpha} \) receptors as described previously (15). In brief, in circular regions of interest, time series were taken with (i) one scan before bleaching and (ii) about 70 iterations of bleaching with 100% laser power (514 nm) followed by 50–100 scans of the bleach region. An image of the whole cell was taken before and after the FRAP time series in order to calculate the loss in total fluorescence of the compartment investigated. This overall loss in fluorescence was taken into consideration to calculate the actual fluorescence recovery and to correct plateau values defining the mobile versus immobile fraction. Mean fluorescence intensities of the bleached region over time were measured using ScionImage\textsuperscript{TM} 4.0.2, and the values were subjected to nonlinear regression analysis using the single exponential association algorithm, \( y = M \times (1 - e^{-Kt}) + b \), where \( b \) represents the relative fluorescence intensity immediately after the bleaching (before recovery), \( M \) is the maximum extent of fluorescence recovery, and \( K \) defines the rate of recovery. Data were weighted by \( 1/y^2 \) to minimize the relative distances squared (which improves the curve fitting for the initial phase of the fluorescence recovery). The half-life of fluorescence recovery \( t_{1/2} \) is defined by 0.69/\( k \), and the plateau, which represents the percentage of mobile receptors, is given by \( M + b \). For a valid comparison of different FRAP experiments, the calculated half-lives of fluorescence recovery were normalized to the length of the bleached segment.

**RESULTS**

*Restricted Collision Coupling of the \( \text{A}_{2\alpha} \) Receptor* 

**Sustained Signaling by the \( \text{A}_{2\alpha} \) Receptor Visualized at the Single Cell Level—**G protein-coupled receptors exist in several conformational states; when activated by agonist, they may engage their cognate G protein(s), or, alternatively, they may undergo desensitization (by the sequential action of specific kinases and recruitment of arrestin and clathrin adapters). These different species may differ in their mobility and thus confound the analysis. In addition, the time scale over which FRAP analysis can be carried out has a lower limit (of \( \geq 50 - 70 \) s; see below). This precludes the detection of dynamic changes, which occur during the initial events of receptor activation, because these occur on the time scale of milliseconds to seconds (16). Thus, experiments have to be done under steady state conditions (i.e. in the presence of antagonist) to force the receptor into the inactive conformation or in the continuous presence of agonist under the assumption that the receptor undergoes productive coupling to \( G_s \) rather than desensitization. We therefore verified that, over the observation periods typically employed in subsequent FRAP experiments, the \( \text{A}_{2\alpha} \) receptor gave rise to sustained G protein signaling; we selected two cell types in which the receptor was endogenously expressed: (i) the rat pheochromocytoma cell line PC12 and (ii) striatal neurons. The cells were transiently transfected with plasmids coding for a YFP-tagged version of the catalytic subunit of protein kinase A and incubated with 0.1 \( \mu \)M CGS21680 or 10 \( \mu \)M XAC for the indicated time. A, cells were monitored under a fluorescent microscope using the three-filter method; the images shown were captured with the FRAP filter set and displayed in pseudocolors to better illustrate the changes of NFRET intensity. Bar, 10 \( \mu \)m. B, cells were stimulated either with CGS21680 (closed circles) or XAC (open squares) as described above. NFRET was calculated as described under “Experimental Procedures”; data points represent means ± S.D. of five cells examined in three independent experiments. Inset, cells were stimulated with 0.1 \( \mu \)M CGS for 10 min, followed by an addition of 10 \( \mu \)M XAC (hatched bar). NFRET is shown from a single cell; the experiment was repeated twice with similar results.

![Figure 1](image-url)
resonance energy transfer declined, reflecting stimulation of adenyl cyclase and the resulting dissociation of the catalytic subunit from the regulatory subunit dimer. It is evident from Fig. 1B that cAMP levels remained elevated for up to 30 min of agonist stimulation. The addition of 10 μM of the antagonist XAC did not result in any change of PKA activity. However, the addition of the antagonist XAC to agonist-stimulated cells restored resonance energy transfer to the original level (Fig. 1B, inset). A similar recovery was also seen upon removal of the agonist by washing (not shown). This ruled out the possibility that the persistent loss of FRET was due to degradation of one of the two fluorescent PKA subunits. Based on these observations, we conclude that Gs was continuously activated by the A2A receptor and that there was no indication for desensitization of adenyl cyclase activation. PC12 cells are a tumor-derived cell line and express high levels of A2A receptors (>1.5 pmol/mg; see Ref. 18). These high receptor levels may titrate out components of the desensitization machinery. We therefore also confirmed this observation in striatal neurons, where the receptor is endogenously expressed (at ~1 pmol/mg, with substantial enrichment in synaptic spines); stimulation of the A2A receptor elicited sustained cAMP accumulation, as judged from the persistent activation of the reporter PKA (Fig. 2A). In contrast, a transient response was observed upon stimulation of D1 receptors, which are also expressed on striatal neurons (albeit on those of the direct pathway). Within 30 min of continuous agonist challenge, resonance energy transfer between the catalytic and regulatory subunit was restored to the original level (Fig. 2B). Thus, the D1 receptor-induced cAMP response had undergone complete desensitization.

The measurement of receptor diffusion and mobility may also be confounded by recycling of receptors from an endocytic pool, which replenishes the receptor at the membrane (and thus spuriously increases fluorescence recovery). We performed antibody feeding experiments to estimate the size of the internalized receptor pool; a FLAG-tagged version of the A2A receptor was expressed in HEK293 cells, and the cells were incubated with the M2 anti-FLAG antibody prior to agonist (CGS21680) or antagonist (XAC) treatment. Thereafter, the cells were challenged with agonist or antagonist for up to 1.5 h and subsequently fixed for immunostaining. Although some intracellular immunoreactivity was visible, which represented internalized A2A receptors, the bulk of the receptors remained at the cell surface at all time points (0, 5, 10, 15, 20, 30, 60, and 90 min) tested. We did not observe a difference between agonist (Fig. 3A, top row) and antagonist-treated cells (Fig. 3A, bottom row). However, antibody feeding may be too insensitive for visualizing rapidly cycling receptors (internalization and subsequent rapid exocytosis), because the limited sample throughput does not allow for resolving closely spaced intervals. As an alternative method that allowed us to visualize internalization in real time, a strategy was employed that relied on the quasisimultaneous confocal visualization of the YFP-tagged A2A receptor and CFP-tagged Rab5 (a marker for early endocytotic vesicles). Again, administration of CGS21680 did not result in any appreciable increase in A2A receptor endocytosis (Fig. 3B). Structures that contained both Rab5 and the A2A receptor were formed at the cytoplasmic leaflet and also pinched off the membrane. However, this phenomenon was not only observed after the addition of CGS21680 but also in the presence of the antagonists ZM241385 or XAC or if cells had been only treated with adenosine deaminase to deplete endogenous adenosine (not shown). Accordingly, this endocytosis was classified as basal internalization of the A2A-adenosine receptor. More importantly, basal internalization only removed a modest fraction of the receptor, and the bulk of the A2A receptors stayed at the cell surface. As a positive control, we used the YFP-tagged CRF receptor-1, which readily internalized in CFP-Rab5-positive vesicles upon agonist stimulation (results not shown). Finally, we also evaluated the capacity of agonist to internalize the A2A receptor endogenously expressed in PC12 cells. Cells were preincubated in the presence (Fig. 3C, left-hand set of bars) or absence of the agonist CGS21680 (Fig. 3C, right-hand set of bars). After removal of agonist, receptor levels were determined by binding of the antagonist [3H]ZM241385. The experimental approach relied on the difference in cell permeability of XAC (lipophilic and hence cell-permeable) and of CGS21680 (a hydrophilic...
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Lateral Mobility of the A<sub>2A</sub> Receptor Is Not Affected by Agonist Exposure—The control experiments described above verified that the bulk of the A<sub>2A</sub> receptor stayed at the cell surface even upon prolonged agonist stimulation. Accordingly, it was possible to measure the lateral mobility of the receptor without confounding effects arising from either receptor desensitization or recycling. A<sub>2A</sub> receptor activation is predicted to result in the transient formation of a ternary complex that is larger in size by a factor of 3 than the receptor alone (i.e. 135 kDa versus 45 kDa) if the receptor interacted as a monomeric species with a G protein heterotrimer. Because of the increased size, this complex is expected to have a reduced mobility, and it should be detectable by FRAP, provided that this complex accumulates under steady state conditions; the YFP-tagged version of the A<sub>2A</sub> receptor was expressed in HEK293 cells, and a membrane strip of 5 μm was bleached using an intense laser beam (Fig. 4A). The lateral diffusion rate of the A<sub>2A</sub> receptor determined the rate of fluorescent recovery in the bleached region (Fig. 4, A and B). The half-life of recovery (t<sub>1/2</sub>) was normalized to the length of the stretch, and the distance (in μm) was divided by the half-life of recovery, which gave a value for the relative speed of lateral movement. If HEK293 cells express moderate to high levels of A<sub>2A</sub> receptor, the receptor is found in an abundant amount in intracellular compartments (10). We therefore selected cells expressing low levels of the receptor (i.e. cells in which the receptor was visualized predominantly at the cell surface).

The second parameter that can be extracted from FRAP measurements is the mobile fraction of a certain protein that corresponds to the maximum fluorescence intensity (corrected for bleaching during repeated measurements) reached after recovery from photobleaching. The lateral mobility of the A<sub>2A</sub> receptor was not modulated upon administration of an agonist or an antagonist compared with basal levels (Fig. 4C); also, the mobile fraction of the A<sub>2A</sub> receptor was not affected, with maximum recovery reaching 55 to 65% of the original fluorescence (Fig. 4D).

Mobility of the Hetero-oligomeric Complex Formed by the D<sub>2</sub>-dopamine/A<sub>2A</sub>-adenosine Receptor—The D<sub>2</sub>-dopamine receptor was analyzed for comparison, because it is a potential hetero-oligomeric partner of the A<sub>2A</sub> Receptor (17); a decreased mobility was observed after the addition of the D<sub>2</sub>-agonist quinpirole but not after the addition of the antagonist haloperidol (Fig. 4E); the mobile fraction of the D<sub>2</sub> receptor remained unchanged under all conditions (Fig. 4F). The mechanistic basis for the agonist-induced decrease in mobility was not further investigated in detail; however, it was contingent on G protein coupling because it was eliminated by pretreating the cells with pertussis toxin (data not shown). We verified that, under the conditions employed, A<sub>2A</sub> receptor and D<sub>2</sub>-dopamine receptor formed hetero-oligomers using two different techniques of FRET microscopy, which relied on bleaching of either the acceptor or of the donor fluorophore. Resonance energy transfer to the acceptor quenches donor emission; accordingly, bleaching of the acceptor leads to increased emission of fluorescence by the donor. Fig. 5A illustrates an example of donor recovery after acceptor photobleaching-FRET microscopy to visualize the interaction of CFP-tagged D<sub>2</sub> receptor (donor) with the YFP-tagged A<sub>2A</sub> receptor (acceptor); the fluorescence...
The mobile fraction was not modulated by the addition of sulpiride. However, A2A receptor ligands did not affect the photobleaching half-time; in contrast, the addition of the D2 agonist quinpirole increased resonance energy transfer, because it afforded protection of the donor against bleaching (Fig. 5B). This may be due to the formation of additional complexes or, alternatively, due to a structural rearrangement in existing heterooligomers. It is not possible to differentiate between these two possibilities by using FRET microscopy. However, A2A receptor ligands did not cause any appreciable change in photobleaching lifetimes (Fig. 5C).

The FRET experiments verified that under the conditions employed, the A2A receptor and the D2 receptor formed hetero-oligomeric complexes in the plasma membrane and that these heterodimers were not disrupted by agonist activation. As shown in Fig. 4, these receptors differed in the extent to which agonists caused changes in receptor mobility. It was therefore of interest to investigate the mobility of the A2A receptor in the presence of the D2 receptor and vice versa. FRAP analysis was performed in HEK293 cells expressing both receptors; we stress that cotransfection of cells did not result in a change in individual receptor levels and that expression efficiency was reasonably comparable for tagged and untagged receptors and for D2 and A2A receptors (not shown). Because the expression of a CFP-tagged receptor may affect FRAP of the YFP-tagged receptor or interfere by bleed-through of fluorescence emission, each receptor was investigated as a YFP-tagged species in the presence of the untagged second receptor. The plasmid coding for the second untagged receptor was added in a 3-fold excess; under these conditions, all cells that express the YFP-tagged receptor can be assumed to express an excess of the untagged receptor. Coexpression of the D2 receptor did not affect the mobility of the YFP-tagged A2A receptor regardless of whether the agonist CGS21680 was used to activate the receptor or whether the inactive conformation was stabilized by he antagonist ZM241385 (Fig. 6A). In contrast, coexpression of the A2A receptor abolished the agonist-induced decrease in mobility of the YFP–tagged D2 receptor completely (Fig. 6C). The mobile fractions were not modulated by the addition of

FIGURE 4. The lateral mobility of the A2A receptor compared with the D2 receptor. A, HEK293 cells were transiently transfected with plasmids coding for the YFP–A2A receptor. A stretch of ~5 μm of the cell membrane (red circle) was bleached with 70 iterations of an intense laser beam, and the recovery of this region was observed over time. Bar, 10 μm. B, the region of interest was selected; the pixel intensity was quantified and plotted over time to determine the extent of recovery and its kinetics. Data were subjected to nonlinear least squares curve fitting using an equation describing a monoexponential rise to a maximum from a basal level. HEK293 cells were transiently transfected with plasmids coding for the YFP–A2A receptor (C and D) or the D2 receptor (E and F); A2A receptor-expressing cells were treated with vehicle, 1 μM CGS21680 (CGS), or 5 μM ZM241385 (ZM); D2 receptor-expressing cells were treated with vehicle, 5 μM haloperidol, or 5 μM quinpirole. The numbers of observations for each condition were between 20 and 30 in at least five independent experiments; 5–10 cells were analyzed in each experiment. Data points (means ± S.D.) were subjected to nonlinear regression analysis to obtain the rate constant k. The calculated constants were converted to the corresponding half-life ([t/2] = ln2/k), and relative mobility (C and E) was calculated, v = μm (bleached region)/t/2. The mobile fraction (D and F) is expressed in percentage of total fluorescence corrected for bleaching losses. Statistical analysis was done by one-way ANOVA followed by Tukey’s post hoc test (**, p < 0.01).
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adenosine or dopamine receptor agonists or antagonists, respectively (Fig. 6, B and D). These changes in receptor mobility cannot simply be explained by changes in the coupling of the D$_2$ receptor to G$_{i}$; in fact, in the presence of the A$_{2A}$ receptor, the D$_2$ receptor still interacts with G$_{i}$ (18), although the complex is less susceptible to modulation by guanine nucleotide (19). Possible interpretations are that (i) agonists not only induce coupling of the D$_2$ receptor to G$_{i}$ but also thereby promote association of this receptor with a different membrane compartment; (ii) in contrast, the A$_{2A}$ receptor is per se located in a different membrane microdomain, where its mobility is lower; (iii) in the hetero-oligomer, the A$_{2A}$ receptor is phenotypically dominant.

Cholesterol Rather than the Actin Cytoskeleton Restricts the Mobility of the A$_{2A}$-adenosine Receptor—Membrane microdomains may be created and stabilized via fencing and picketing by the actin cytoskeleton. The carboxyl terminus of the A$_{2A}$ receptor has been reported to interact with the actin-binding protein α-actinin, and the interaction domain was mapped within amino acids 293–321 of the A$_{2A}$ receptor, which is within the membrane-adjacent portion of the C-tail (20). Accordingly, truncated mutants of the A$_{2A}$ receptor were used to determine whether the C terminus restricted receptor mobility. The two mutated versions, A$_{2A}$ receptor-(1–360) and A$_{2A}$ receptor-(1–311), were truncated by 52 amino acids and 101 amino acids, respectively. FRAP experiments showed that the truncations of the C-tail did not result in any difference in receptor mobility or its mobile fraction (Fig. 7, A and B). In the A$_{2A}$ receptor-(1–311), the α-actinin binding site ought to be compromised, but its precise location is not known (20). Thus, we cannot formally rule out that this truncated version is still capable of interacting with the actin cytoskeleton. Accordingly, we disrupted the cortical F-actin by pretreating the cells for 1 h with 1 μM latrunculin A, an inhibitor of actin polymerization (21, 22). However, the mobility of the A$_{2A}$-adenosine receptor was not altered (Fig. 7C). These observations support the conclusion that any possible interaction of the A$_{2A}$ receptor with the actin cytoskeleton does not translate into a major effect on the lateral mobility of the receptor. Disruption of cortical F-actin caused a decline by on average 10% in the mobile fraction of the receptor, and this difference was statistically significant (Fig. 7D). Although this finding is difficult to interpret mechanistically, it is inconsistent with actin-mediated restricted mobility. Taken together, the experiments summarized in Fig. 7 did not provide any evidence for a major role of the cortical actin cytoskeleton in confining the A$_{2A}$ receptor to actin-scaffolded microdomains. We also examined the alternative explanation, namely that the slow mobility of the A$_{2A}$ receptor was determined by the lipid composition of the plasma membrane. Two distinct types of microdomains have been described: cholesterol-rich domains and domains enriched with glycosphingolipids. Cholesterol was sequestered in the plasma membrane by pretreating cells with the polyanionic dextrin D-PDMP. Cholesterol depletion/sequestration increased the mobility of the A$_{2A}$ receptor compared with untreated cells in both the agonist- and the antagonist-liganded conditions (Fig. 8A). The increase in receptor mobility observed after methyl-β-cyclodextrin treatment was not as prominent as that seen after treatment with filipin III but nevertheless statistically significant. However, the mobile fraction of the A$_{2A}$ receptor was decreased only after the addition of methyl-β-cyclodextrin (Fig. 8B). Synthesis of glycosphingolipids can be inhibited by an analogue of ceramides, d-PDMP, which blocks UDP-glucose:N-acylsphingosine glucosyltransferase (23). We verified that d-PDMP did affect HEK293 cells by monitoring the rapid trafficking of VSV-G, which is contingent on the presence of sphingolipids (24). At the concentration employed (30 μM), d-PDMP substantially delayed cell surface
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...modestly but significantly reduced.

0.42 pmol/mg protein and 3.0 depletions of cholesterol did not affect binding parameter esti-
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with filipin III in a manner identical to the conditions outlined for FRAP experiments. Sequestration of cholesterol completely abolished the CGS21680-induced elevation of cAMP (Fig. 10A, fourth bar). Cholesterol depletion did not interfere with adenyl cyclase activity per se; forskolin still activated adenyl cyclase directly in filipin III-treated cells (Fig. 10A, fifth bar). The readdivision of cholesterol by using water-soluble cyclodextrin complexes restored receptor-mediated cAMP accumulation (not shown). Finally, a different Gs-coupled receptor, the CRF2 receptor, was much less susceptible to the action of filipin III; agonist-induced cAMP accumulation was sustained in filipin III-treated HEK293 cells heterologously expressing CRF2 receptors (Fig. 10B). The A2A-adenosine receptor can also stimulate the ERK/MAPK pathway in a manner independent of Gs (25, 26) but dependent on ARNO (27). Filipin III-treated cells were stimulated with the agonist CGS21680, and the time course of MAPK/ERK stimulation was monitored by immunoblotting for the phosphorylated (i.e. active) forms of the protein (Fig. 10C). Cholesterol sequestration did not affect stimulation of ERK/MAPK, and the biphasic response activity was maintained (Fig. 10C, bottom). These results clearly indicated that the A2A receptor required cholesterol in the plasma membrane to interact fruitfully with Gs to thereby activate cAMP synthesis but not for signaling via the ERK/MAPK pathway.

**DISCUSSION**

Although many basic tenets of the original fluid mosaic model of the lipid bilayer (1) are still considered to be valid, the model has been substantially modified in the past 4 decades to account for experimental findings; one important conceptual change was the lipid raft model, which posits a compartmentalized distribution of lipids based on the differential miscibility of glycerophospholipids, sphingolipids, and cholesterol (28). Compartmentalization further occurs through anisotropic distribution of membrane proteins within these lipid domains and via interaction with the cytoskeleton. The most important finding of the present work is the observation that cholesterol-rich membrane microdomains are essential for supporting specifically one signaling cascade of the A2A-adenosine receptor, namely activation of adenyl cyclase via Gs. In contrast, A2A-receptor-dependent stimulation of MAPK is not affected by cholesterol depletion. These two signaling pathways were previously shown to be independent; specifically, MAPK stimulation was shown to be independent of Gs (25, 26) and presumably of all other heterotrimeric G proteins (29) and to rely on the recruitment of ARNO (27). The present observations are consistent with the interpretation that activation of the two signaling pathways is physically segregated. The size of the receptor is small by comparison with the heterotrimeric G protein; similarly, ARNO is larger in size than the tightly packed G protein-coupled receptor. Thus it is unlikely that the A2A receptor can simultaneously bind Gs and ARNO, and there must be rules for an ordered engagement of these two (and additional) interaction partners (30). The fact that activation of Gs and the interaction with ARNO may take place in different places provides a solution to this conundrum. To the best of our knowledge, the A2A-adenosine receptor is the first G protein-coupled receptor shown to discriminate between cAMP signaling and MAPK signaling in a manner dependent on the lipid environment of the membrane, requiring cholesterol for one cascade but not for the other.
Fatty acylation, particularly palmitoylation, can act as a targeting signal for partitioning proteins into rafts (31). G proteins were originally proposed to be enriched in lipid rafts because of their lipid modifications (i.e. N-terminal myristate and palmitate on Gs is sufficient to partition Gs into rafts) (32). Isoprenoid modifications like farnesylation and geranylgeranylation are not favorable for association with rafts (32). Gs is palmitoylated, and this favors association with rafts (33), and a second palmitoylation in the N terminus of Gs sensitized Sf9 insect cells up to 200-fold for adenylyl cyclase-stimulating agents (34). Taken together, these findings argue for a model, where Gs-dependent activation of adenylyl cyclase occurs in lipid rafts. The present observations are also consistent with this interpretation, because the A2A receptor required cholesterol and thus presumably intact cholesterol-rich domains for G protein coupling and adenylyl cyclase activation. Many GPCRs are also found to a variable extent in lipid rafts (reviewed in Ref. 35). The extent to which proteins are correctly assigned to lipid rafts or specialized microdomains has been subject to criticism (36). The debate centers mainly on the validity of experimental procedures used to identify these microdomains. Their nanometer diameter (25–100 nm) is beyond the resolution of light microscopy (~200 nm). Historically, lipid raft-associated proteins were defined as those resistant to extraction with Triton X-100 or related detergents. These detergent-resistant membrane fractions have a low buoyant density and thus can be separated by sucrose density gradient centrifugation. This method is thought to be prone to many artifacts: the ratio of protein to detergent, the nature of the detergent, the temperature, and the extraction conditions are key parameters and can greatly influence the outcome (37). A recent review (35) lists 22 GPCRs that are thought to be associated with detergent-resistant membrane fractions or caveolae. Some GPCRs are almost exclusively located in detergent-resistant membranes (e.g. the gonadotropin-releasing hormone receptor) (38), whereas others are only present in small amounts (e.g. the oxytocin receptor) (39). The localization signals for GPCRs to partition into rafts are not yet understood. Both modifications of the intracellular loops or C termini and the interaction of the transmembrane domains with cholesterol or gangliosides may be involved in targeting receptors to lipid rafts. It is unclear what determines the association of the A2A receptor with cholesterol-rich rafts. Most importantly, the A2A receptor is not palmitoylated, because it lacks the canonical cysteine residue at the end of helix-8 (the juxtamembrane portion after the seventh transmembrane helix). The observations presented in this work provide circumstantial evidence in support of the conjecture that the hydrophobic core of the receptor suffices to trap the receptor in the cholesterol-rich areas.

The second major finding in this work sheds light on the coupling mode of the A2A2-adenosine receptor to Gs. In theory, a receptor is predicted to move over the cell surface and to randomly collide with G proteins. In the absence of agonist, this random collision is unlikely to result in any signal propagation. If the receptor is activated, the collision is thought to result in a fruitful interaction (i.e. G protein activation). The receptor needs to select for its cognate G protein and hence discriminate between closely related G proteins; this is presumably achieved by kinetic proofreading (40); if the receptor collides with a G protein other than its cognate G protein(s), a productive ternary complex cannot form. The interaction may be greatly

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**FIGURE 8.** The mobility of the A2A receptor is enhanced by removal/sequestration of cholesterol from the plasma membrane. HEK293 cells transiently expressing the YFP-A2A receptor were treated with 1 μM CGS21680 (CGS) or 5 μM ZM241385 (ZM) as indicated. Cells were pretreated with vehicle, 5 μg/ml filipin III for 20 min, or 10 mM methyl-β-cyclodextrin (CD) for 90 min (A and B) or 30 μM PDMP for 48 h (C and D). FRAP analysis was performed as described in the legend to Fig. 3 and under “Experimental Procedures.” Mobility (A and C) and the mobile fraction (B and D) were calculated as outlined in Fig. 4 and under “Experimental Procedures.” Values represent means ± S.D. from between 15 and 25 observations from at least three independent experiments recording 5–10 cells each. The statistical analysis was done by a one-way ANOVA followed by Tukey’s post hoc test (*, p < 0.05; **, p < 0.01; ***, p < 0.001).
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FIGURE 9. Cholesterol depletion impairs G protein coupling of the $A_{2A}$ receptor. A, HEK293 cells stably expressing the $A_{2A}$ receptor were treated with either vehicle (closed circles) or 5 µg/ml filipin III (inverted triangles) for 20 min prior to and during the experiment. Cells (5 x 10^4/reaction) were incubated with 8 units/ml adenosine deaminase and increasing concentrations of [^3H]ZM241385 for 60 min in 0.5 ml of medium and subsequently filtered through Whatman GF/B filter paper; nonspecific binding determined in the presence of 10 µM CGS21680 was <10% of total binding at the highest radioligand concentration employed and was subtracted. Data are from one representative experiment carried out in duplicate, which was repeated three times with similar results. B and C, cells treated with either vehicle (B) or 5 µg/ml filipin III (C) and incubated with 4 nM[^3H]ZM241385 and increasing concentrations of CGS21680 in the absence (closed circles) or presence (inverted triangles) of GTPγS (100 µM). GTPγS-treated cells had been permeabilized by a freeze-thaw cycle (which did not affect total binding). Nonspecific binding determined in the presence of 10 µM XAC was <10% of total binding and was subtracted. Data represent mean ± S.D. of three independent experiments carried out in duplicate.

enhanced, and signal transduction may be rendered more efficient by keeping components in close spatial proximity by assembling signaling complexes tethered to scaffolding proteins or by concentrating the interaction partners in membrane microdomains (41). The $A_{2A}$ receptor has long been known to undergo restricted collision coupling (3, 4), but neither the mechanistic nor the structural basis for this phenomenon was understood. In the present work, restricted collision coupling was reexamined in order to understand its relation to receptor mobility. The major finding was the observation that the addition of the agonist did not affect the mobility, whereas the mobility of the dopamine $D_2$ receptor was reduced upon agonist binding. It cannot be ruled out that the formation of $A_{2A}$ receptor-G protein complexes is not detectable, because they may rapidly dissociate upon G protein loading and may thus not accumulate to a sufficient extent at steady state for detection. However, it is worth pointing out the following. (i) High affinity agonist-receptor-G protein complexes were observed in intact cells with the $A_{2A}$ receptor; thus, there was a fraction of the receptors that did exist as agonist-receptor-G protein within the intact cell. (ii) In addition, the agonist-induced decrease in the mobility of the $D_2$ receptor was abolished in cells pretreated with pertussis toxin; thus, agonist-induced changes in mobility were in principle detectable, and they were contingent on G protein coupling. (iii) Finally, in cells co-expressing the $D_2$ and the $A_{2A}$ receptor, the $D_2$-agonist failed to induce any decrease in receptor mobility. Because these two receptors form a heteromeric complex (17), this finding indicates that the $A_{2A}$ receptor is phenotypically dominant in the heteromeric complex. Thus, the $D_2$ receptor is likely to be recruited into the lipid domain surrounding the $A_{2A}$ receptor.

In random collision coupling, a receptor can in principle activate the full complement of G proteins on the cell surface. In contrast, in restricted collision coupling, the receptor is confined to a certain area of the membrane and only interacts with G proteins in its vicinity. Regulating receptor number has different consequences in the two coupling modes; in random collision coupling, increased surface expression levels translate into a leftward shift of the agonist concentration-response curve. In other words, the large number of receptors results in increased sensitivity to the agonist; occupancy of a minute fraction of the receptors suffices to elicit the maximum agonist-induced response, because there is a large receptor reserve. This was, for instance, observed for the $B_2$ adrenergic receptor, where overexpression dramatically lowered the $EC_{50}$ of agonists (42). In the restricted collision coupling model, the prediction is different. Agonist sensitivity (i.e. $EC_{50}$) cannot be affected by raising receptor levels, but the magnitude of the response (i.e. $V_{max}$) must increase (5). In fact, this has been previously observed in the very same cells employed here (HEK293). Raising the number of $A_{2A}$ receptors at the cell
surface increased \( V_{\text{max}} \) substantially, but EC\(_{50} \) did not change (10). Similarly, if the receptor number was reduced by 90% through a covalent modification, the \( V_{\text{max}} \) of adenylyl cyclase activation declined, but the EC\(_{50} \) was only marginally affected (43). These considerations further support the conjecture that the restricted collision coupling model is a feature of the A\(_{2A} \) receptor proper, which is observed regardless of the cellular background. Taken together, the current observations substantiate the restricted collision coupling model and allow for a mechanistic interpretation; the receptor is confined to specific domains, in which its mobility is restricted. Accordingly, if these domains are depleted of cholesterol and thus membrane fluidity is augmented, mobility increases substantially. However, if the receptor is not confined to the cholesterol-rich microdomains, it cannot efficiently recruit and activate \( G_o \). This interpretation is supported by three lines of evidence: (i) cholesterol depletion promoted receptor mobility but (ii) precluded the formation of receptor-\( G \) protein complexes and (iii) blunted agonist-induced cAMP elevation.

The technology used to measure the lateral mobility of the receptor has several limitations that need to be taken into account. (i) The measurement of fluorescent recovery only provides data for population averages; (ii) changes in complex size may be too subtle to allow for detection; and (iii) there is a lower limit on the time scale at which effects may be monitored. This is determined by the recovery lifetime; in the present experiments, bleaching and recovery took a minimum of 70 s. Thus, rapid changes in conformation that transiently alter mobility may escape detection. Last, it has to be mentioned that (iv) the tag may per se affect the mobility. It may be argued that the observations are confounded by the fact that the YFP was placed at the C terminus; this may disrupt the interaction with a PDZ-domain protein or a related protein, which is responsible for tethering the receptor to specific sites. Although this cannot be formally ruled out, it is worth pointing out that many interactors have been identified that bind to the A\(_{2A} \) receptor (30), none of which have been found to require the last 3 amino acids.\(^5\)

Taken together, these arguments indicate that the caveats do not invalidate the present findings. Most importantly, the most striking finding is unrelated to the presence or absence of the YFP moiety on the C terminus of the receptor. Cholesterol depletion also abolished A\(_{2A} \) agonist-induced cAMP accumulation but did not preclude MAPK stimulation if the assays were done with an A\(_{2A} \) receptor that did not carry any fluorescent protein on its C terminus. The spatial separation of the two signaling cascades activated by the A\(_{2A} \)-adenosine receptor may also have an impact on our understanding of how the A\(_{2A} \) receptor signals in the synapse. Synapse formation is contingent on the presence of cholesterol, which is in part supplied by the glia (44). Postsynaptic specializations are stabilized by cholesterol (45, 46). Conversely, upon activation, ARNO and ARF6 suppress dendritic sprouting and branching (47). Thus, it is attractive to speculate that the level of cholesterol may have an impact on which signaling pathway is preferentially utilized by postsynaptic A\(_{2A} \) receptors in the striatum.

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\(^5\) O. Kudlacek and M. Freissmuth, unpublished observations.
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