The Adenosine A2A Receptor Interacts with the Actin-binding Protein α-Actinin*

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Recently, evidence has emerged that heptaspanning membrane or G protein-coupled receptors may be linked to intracellular proteins identified as regulators of receptor anchoring and signaling. Using a yeast two-hybrid screen, we identified α-actinin, a major F-actin-crosslinking protein, as a binding partner for the C-terminal domain of the adenosine A2A receptor (A2AR). Colocalization, co-immunoprecipitation, and pull-down experiments showed a close and specific interaction between A2AR and α-actinin in transfected HEK-293 cells and also in rat striatal tissue. A2AR activation by agonist induced the internalization of the receptor by a process that involved rapid β-arrestin translocation from the cytoplasm to the cell surface. In the subsequent receptor traffic from the cell surface, the role of actin organization was shown to be crucial in transiently transfected HEK-293 cells, as actin depolymerization by cytochalasin D prevented its agonist-induced internalization. A2A-ICTN, a mutant version of A2AR that lacks the C-terminal domain and does not interact with α-actinin, was not able to internalize when activated by agonist. Interestingly, A2A-ICTN did not show aggregation or clustering after agonist stimulation, a process readily occurring with the wild-type receptor. These findings suggest an α-actinin-dependent association between the actin cytoskeleton and A2AR trafficking.

Adenosine is a molecule that plays a regulatory role in the nervous system, acting presynaptically, postsynaptically, and/or non-synaptically (1). Adenosine mediates its actions through activation of specific G protein-coupled heptaspanning membrane receptors, for which four subtypes have been identified (A1R, A2AR, A3R, and A3R) (2). A2ARs are coupled mostly to Gs proteins (3), thus being mainly linked to adenyl cyclase activation. The role of A2AR in the control of neurotransmitter release has been studied in several brain regions as well as in the peripheral nervous system (4). Compared with the other adenosine receptor subtypes, A2ARs are specially concentrated in the striatopallidal complex (5). Using ultrastructural techniques, Hettinger et al. (6) have recently demonstrated that A2ARs are mostly localized postsynaptically in dendrites and dendritic spines of rat striatal GABAergic (where GABA is γ-aminobutyric acid) neurons. The membrane expression and localization of A2AR are the result of ligand-mediated clustering, internalization, and receptor trafficking (7).

α-Actinin is a major F-actin-cross-linking protein present in both muscle and non-muscle cells. There are four α-actinin genes: two non-skeletal muscle isoforms (α-actinin-1 and -4) and two skeletal muscle isoforms (α-actinin-2 and -3) (8), all of which share a general structure, which can be divided into three functionally distinct domains: the N terminus, which contains two calponin homology domains and which mediates the interaction with actin; a central region composed of four spectrin-like motifs; and the C terminus, which contains EF-hand domains. Recently, the spatial expression of α-actinin-2 in the rat central nervous system was analyzed, revealing that the highest levels of the protein are found in the striatum, cortex, and hippocampus, where it has been shown to interact with the glutamate NMDA receptor (9).

In this study, using a yeast two-hybrid approach and complementary biochemical techniques, we provide evidence for the existence of a physical interaction between A2AR and α-actinin. Furthermore, we also demonstrate that there is a functional association between the actin cytoskeleton and A2AR and more specifically with the receptor internalization process and its membrane agonist-induced aggregation or clustering.

EXPERIMENTAL PROCEDURES

Plasmid Constructs

Two EcoRI-EcoRI fragments of the C-terminal tail of A2AR were subcloned into the bait vector pHybLexA/Zeo (Invitrogen) and the bacterial expression vector pGEX-4T-1 (Amersham Biosciences). One fragment coding for amino acids 293–412 (L2A2) was amplified using Taq DNA polymerase (Sigma) and the following primers: FLA2A (5′-TAA-
The FLAG epitope was introduced between amino acids 6 and 7 of human AGACTAGTAGGCGTAGATGAAGGG-3 [59x652] 7.0). A small amount of glass beads (425 30 30% sample, and the mixture was incubated at 37°C for 1 h with DMEM containing 10% heat-inactivated FCS and 50 μg/ml gentamycin). Trypsinization was stopped by addition of 50 mM Tris-HCl, pH 7.4, containing 1% (v/v) of ice-cold RIPA buffer; and once with 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 1 mM EDTA. Then, 50 μl of SDS-PAGE sample buffer was added to each sample. Bound proteins were dissociated and heated at 37°C for 1 h and resolved by SDS-PAGE on 7.5% gels as described above.

Antibodies

An antiserum against the GST fusion protein containing amino acids 322–412 (SAZA) of A2AR (designated as anti-CTA2A) was used in this study. Immunization of rabbits and affinity purification of the antiserum were performed as described previously (12, 13). Other primary antibodies used were as follows: anti-calnexin monoclonal antibody (Transduction Laboratories), anti-FLAG monoclonal antibody (clone M2; Sigma), anti-α-actinin monoclonal antibody (Sigma), and an anti-α-actinin polyclonal antibody (Santa Cruz Biotechnology), anti-α-actinin monoclonal antibody (Sigma), and anti-β-arrestin polyclonal antibody Ab186 (1:1000 dilution) (14). The secondary antibodies used were as follows: horseradish peroxidase-conjugated goat anti-rabbit IgG (1:60,000 dilution; Pierce), horseradish peroxidase-conjugated rabbit anti-mouse IgG (1:2000 dilution; Dako Corp.), Alexa 488-conjugated goat anti-mouse IgG (1:1000 dilution) and Texas Red-conjugated goat anti-rabbit IgG (1:2000 dilution; both Molecular Probes, Inc.), and Cy3-conjugated donkey anti-mouse IgG (1:250 dilution; Jackson ImmunoResearch Laboratories, Inc.).

Immunoprecipitation

Transfected HEK-293 cells were solubilized with ice-cold RIPA buffer, and lysates were incubated with anti-FLAG monoclonal antibody (2 μg/ml) or anti-CTA2A polyclonal antibody (2 μg/ml) for 2 h. Then, 40 μl of a suspension of 0.1% (w/v) or 0.1% (w/v) of protein A cross-linked to agarose beads (Sigma) was added, and the mixture was incubated overnight with constant rotation at 4°C. The beads were washed twice with ice-cold RIPA buffer; twice with 0.1% (v/v) of ice-cold RIPA buffer; and once with 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 1 mM EDTA. Subsequently, 50 μl of SDS-PAGE sample buffer was added to each sample. Immunoprecipitates were resolved on 7.5% or 10% SDS-polyacrylamide gels as described above.

Biotinylation of Cell-surface Proteins

HEK-293 cells transiently transfected with A2AR-FLAG or A2AR-CTR-FLAG were washed three times with borate buffer (10 mM H3BO3, pH 8.8, and 150 mM NaCl) and then incubated with 50 μg/ml sulfo-NHS-SS-biotin (Pierce) in borate buffer for 5 min at room temperature. Cells were washed three times with borate buffer and again incubated with 50 μg/ml sulfo-NHS-Ssuccinimidyl 6-biotinamidohexanoyl (Pierce) in borate buffer for 5 min at room temperature. Cells were washed three times with borate buffer and again incubated with 50 μg/ml sulfo-Ssuccinimidyl 6-biotinamidohexanoyl in borate buffer for 10 min at room temperature, and 100 mM NH4Cl was added for 5 min to quench the remaining biotin. Cells were washed with Tris-buffered saline, disrupted with three 10-s strokes in a Polytron, and resuspended at 14,000 rpm for 20 min. The pellet was solubilized in ice-cold lysis buffer for 30 min and centrifuged at 14,000 rpm for 20 min. The supernatant was incubated with 80 μl of streptavidin-agarose beads (Sigma) for 1 h with constant rotation at 4°C. Beads were washed twice with ice-cold lysis buffer, twice with

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0.1% (v/v) of ice-cold lysis buffer, and once with ice-cold phosphate-buffered saline. The complexes were dissociated by adding 50 μl of SDS-PAGE sample buffer, heated at 37 °C for 1 h, and then resolved by SDS-PAGE on 10% gels. The gels were run and immunoblotted as described above.

**Immunocytochemistry**

Transiently transfected HEK-293 cells or rat striatal primary cultures were fixed in 4% paraformaldehyde for 15 min and washed with phosphate-buffered saline containing 20 mM glycine (buffer A) to quench the remaining free aldehyde groups. Cells were permeabilized with buffer A containing 0.2% Triton X-100 for 5 min. Blocking was done using buffer A containing 1% bovine serum albumin (buffer B). Cells were labeled for 1 h at room temperature with the indicated primary antibody, washed for 30 min with buffer B, and stained with the corresponding secondary antibody for 1 h. Coverslips were rinsed for 30 min with buffer B and mounted with Immuno Fluore mounting medium (ICN). An Olympus FV 300 scanning laser confocal microscope was used for observations.

**RESULTS**

Yeast Two-hybrid Screening—To identify intracellular proteins interacting with A2AR, the last 120 amino acids of A2AR were fused in-frame with LexA in the pHybLex/A vector (pHYB) (Fig. 1A) and used to screen a mouse brain cDNA library with the yeast two-hybrid system. Of the 24 clones (from the 2 × 10^6 total transformants screened that were found to grow on nutritionally deficient plates and activated the β-galactosidase assay), seven were identified as different isoforms of the actin-binding protein α-actinin: three independent clones for α-actinin-1, another three for α-actinin-4, and one clone for α-actinin-2. To discard a false-positive interaction, all four different α-actinin isoforms were transformed again with LA2A and plated along with different negative and positive controls. As shown in Fig. 1, only those transformants containing the C-terminal tail of A2AR and any of the α-actinins could grow on deficient medium and turned blue in the β-galactosidase assay, as did the positive control dystrobrevin/syntrophin (DB/SYN) (15). To determine the region of the C-terminal domain of A2AR that interacts with α-actinin, another LexA fusion protein of the last 91 amino acids of the receptor was constructed (SA2A) (Fig. 1B) and tested for its ability to bind α-actinin. This shorter fusion protein could not interact with any of the α-actinins (Fig. 1, B and C), thus mapping the interacting domain within amino acids 293–321 of A2AR. The interaction of both the LA2A and SA2A constructs and α-actinins was quantitated using a liquid β-galactosidase assay as described under “Experimental Procedures.” Data are means ± S.E. of three replicates. ACT1, ACT2, ACT3, and ACT4, α-actinin-1, -2, -3, and -4, respectively; DB, α-dystrobrevin; SYN, syntrophin; pHYB, pHybLex; pYES, pYESTrp2; 7TM, seven-transmembrane domain.
against the FLAG epitope and these immunoprecipitates were pulled down with GST-LA2A, but not with GST-SA2A or GST alone. α-Arrestin was detected using a polyclonal antibody against α-arrestin (1:500 dilution). The primary bound antibody was detected using a horseradish peroxidase-conjugated goat anti-rabbit antibody (1:60,000 dilution). The lower panel shows the relative amounts of GST, GST-SA2A, and GST-LA2A fusion proteins used in the pull-down experiment stained with Coomassie Blue.

100 kDa corresponding to amino acids 293 and 321 as the α-arrestin-interacting domain.

Interaction of A2AR and α-Arrestin in Transfected HEK-293 Cells—The association of A2AR and α-arrestin was subsequently studied in transfected HEK-293 cells by double immunolabeling and co-immunoprecipitation experiments. By confocal microscopy analysis of HEK-293 cells transfected with the cDNA coding for A2AR-FLAG, a marked overlap in the distribution of the two proteins was found (Fig. 3A). Interestingly, when the double immunolabeling experiment was performed in HEK-293 cells transiently transfected with the cDNA coding for A1R-FLAG (another adenosine receptor subtype with a shorter C-terminal tail) or with the cDNA coding for a mutant of A2AR lacking the whole C-terminal domain (A2ARCTR-FLAG), no colocalization with α-arrestin was observed (Fig. 3A), suggesting a specificity for the colocalization between A2AR-FLAG and α-arrestin.

When cell extracts of HEK-293 cells transiently transfected with A2AR-FLAG were immunoprecipitated with an antibody against the FLAG epitope and these immunoprecipitates were analyzed by Western blotting using an antibody against α-arrestin, a band of 100 kDa corresponding to α-arrestin was observed (Fig. 3B, lane 4). Interestingly, this band did not appear in immunoprecipitates from cells transfected with the cDNAs coding for LacZ (Fig. 3B, lane 1), A1R-FLAG (lane 2), and A2ARCTR-FLAG (lane 3). These results demonstrate that α-arrestin can interact with full-length A2AR; that the interaction is mediated by the C-terminal tail of A2AR; and that the A2AR/α-arrestin interaction is receptor subtype-specific, as A1R did not interact with α-arrestin.

Interaction of A2ARs with α-Arrestin in Rat Striatal Homogenates and Primary Rat Striatal Neurons—To assess the physiological relevance of the A2AR/α-arrestin interaction, we performed co-immunoprecipitation experiments with rat striatal homogenates and double immunolabeling with primary cultures of rat striatal neurons. Using soluble extracts from rat striatum that had been shown by Western blotting to contain both α-arrestin and A2AR (Fig. 4A, lane 1), anti-CT2A2A antibody could immunoprecipitate a band of ~100 kDa that was detected with α-arrestin antibody (lane 2). This band did not appear when protein A-agarose was used for immunoprecipitation (Fig. 4A, lane 3), showing that there was no unspecific interaction between α-arrestin and the resin.

The distribution of α-arrestin and A2AR in primary rat striatal neurons was also tested. By confocal microscopy analysis, a similar punctate distribution and a partial colocalization for both proteins were found (Fig. 4B). Colocalization was not
general, but it occurred both in the cell body, where the degree of colocalization of A2AR with α-actinin was very high (Fig. 4B, arrows), and at specific aggregates in dendrites (arrowheads). These observations are consistent with the idea that α-actinin and A2AR associate in striatal neurons.

Correlation between α-Actinin Binding and A2AR Ligand-induced Trafficking—Because of the role of α-actinin in organizing and cross-linking actin filaments, we analyzed the possibility that the interaction between α-actinin and A2AR reported in this study could be related to the trafficking of the receptor, both its clustering and internalization processes occurring upon agonist stimulation. Although the agonist CGS-21680 (2-ethylcarboxyethyl)phenethylamino-5'-N-ethylcarboxyamidoadenosine) produces a marked internalization of A2AR (7), the molecules involved in this process are not well known.

For example, the role of β-arrestin proteins in the internalization process of A2AR has not yet been described. Confocal scanning microscopy was used to visualize in cotransfected cells the possible translocation of β-arrestin-1-GFP or β-arrestin-2-GFP from the cytoplasm to the cell surface. Fig. 5A shows that, in the absence of agonist, β-arrestin-1-GFP was homogeneously localized in the cytosol of cells also expressing A2AR-FLAG. Upon stimulation with 200 nM CGS-21680, a rapid (2 min) translocation of β-arrestin-1-GFP to the cell surface was observed. Similar results were obtained when A2AR was cotransfected with β-arrestin-2-GFP (data not shown).

To further examine whether A2AR internalization is arrestin-dependent, the receptor was cotransfected with β-arrestin-1(S412D), a mutant with dominant-negative properties with respect to receptor internalization (16). To analyze the amount of receptor in the plasma membrane, we isolated cell-surface proteins by protein biotinylation using a membrane-impermeant biotin ester. Proteins isolated by streptavidin-agarose affinity precipitation were analyzed by SDS-PAGE and immunoblotted using affinity-purified anti-CTA2A polyclonal antibody (1:200 dilution) and anti-α-actinin monoclonal antibody (1:100 dilution). Cells were analyzed by double immunofluorescence with a confocal microscope. Superimposition of images revealed colocalization (yellow) both in the cell body (arrows) and in dendrites (arrowheads). Scale bar = 10 μm.

Fig. 4. In vivo interaction of A2AR and α-actinin in rat striatum. A, solubilized extracts from rat striatum were subjected to immunoprecipitation analysis using anti-CTA2A antibody (2 μg/ml). Extracts (lane 1) and immunoprecipitates (lanes 2) were analyzed by SDS-PAGE and immunoblotted (IB) using anti-α-actinin polyclonal antibody (1:500 dilution) (upper panel) and the polyclonal antibody against A2AR, anti-CTA2A antibody (Ab; 1:2000 dilution) (lower panel). Both A2AR and α-actinin could be detected in the crude extract (lane 1) and in the immunoprecipitate (lane 2) with anti-CTA2A antibody, but they could not be detected with protein-A agarose alone (lane 3).

B. primary cultures of rat striatal neurons (days in vitro 14–21) were cultured and processed for immunolabeling as described under “Experimental Procedures” using affinity-purified anti-CTA2A polyclonal antibody (1:50 dilution) and anti-α-actinin monoclonal antibody (1:100 dilution). Cells were analyzed by double immunofluorescence with a confocal microscope. Superimposition of images revealed colocalization (yellow) both in the cell body (arrows) and in dendrites (arrowheads). Scale bar = 10 μm.

Fig. 5. Ligand-induced A2AR internalization. A, HEK-293 cells transiently transfected with A2AR-FLAG (A2A-FLAG) plus β-arrestin-1-GFP (β-arrestin-1-GFP) were treated with vehicle (Control) or 200 nM CGS-21680 for 2 min (2 min) at 37 °C. Then, cells were fixed as described under “Experimental Procedures” and labeled with anti-FLAG monoclonal antibody (1:2000 dilution) and with anti-α-actinin polyclonal antibody. Immunoblots (upper panel) and immunoprecipitates (lower panel) were analyzed by SDS-PAGE and immunoblotted using anti-CTA2A polyclonal antibody (1:2000 dilution) and anti-α-actinin polyclonal antibody (1:500 dilution)

B. HEK-293 cells transiently transfected with A2AR-FLAG ((lanes 1 and 2) or A2AR-FLAG plus β-arrestin-1(S412D) (βarr1 S412D)) (lanes 3 and 4) were treated with vehicle (lanes 1 and 3) or with 200 nM CGS-21680 (lanes 2 and 4) for 2 h at 37 °C. Cell-surface labeling was performed as described under “Experimental Procedures.” Crude extracts and biotinylated proteins were subsequently analyzed by SDS-PAGE and immunoblotted using anti-CTA2A polyclonal antibody (1:2000 dilution), anti-β-arrestin polyclonal antibody Ab186 (1:1000 dilution), and anti-calnexin monoclonal antibody (1:250 dilution).
incubation for 1 h at 37 °C with fresh medium (Fig. 6, A and C, lane 3). The results show that the amount of receptor present in the cell surface decreased substantially in cells treated with CGS-21680 (Fig. 6A, lane 2) compared with control cells (lane 1). When the agonist was washed out, the cell-surface expression of the receptor was partially recovered after 1 h (Fig. 6A, lane 3). In contrast, pretreatment of cells with cytochalasin D avoided agonist-induced internalization of A2AR (Fig. 6C, lane 2). Quantitation of the cell surface-associated receptor by densitometric scanning of the immunoreactive bands on the films indicated that agonist treatment or challenge induced an internalization of 74% of the total cell-surface receptor (Fig. 6D).

Under the same conditions, when cells were pretreated with the actin-disrupting agent cytochalasin D, the agonist challenge did not induce internalization of the receptor (Fig. 6D). These results indicate that a completely organized actin cytoskeleton is necessary for the agonist-dependent internalization of A2AR. Moreover, the internalization ability of A2ΔCT2AR, the C-terminal deletion mutant of A2AR that showed neither colocalization nor co-immunoprecipitation with α-actinin (Fig. 3), was tested. After agonist treatment of transiently transfected cells with A2ΔCT2AR-FLAG, the membrane proteins were isolated (see above) and analyzed by immunoblotting using anti-FLAG antibody to detect A2ΔCT2AR-FLAG (Fig. 7). The results show that the amount of receptor present at the plasma membrane remained invariant after agonist treatment (Fig. 7, lane 2) compared with the untreated cells (lane 1). Again, the absence of calnexin in the streptavidin isolates indicated that the biotin could not penetrate the cell (Fig. 7, lower panels).

Proper attachment of dopamine D2 receptors to the actin cytoskeleton through the actin-binding protein filamin A has been found to affect cell-surface clustering of this receptor (20). To analyze the plasma membrane expression pattern of A2AR as a function of its ability to bind α-actinin, immunofluorescence experiments with A2AR-FLAG- and A2ΔCT2AR-FLAG-transfected HEK-293 cells were performed. A2AR-FLAG was shown to be uniformly expressed at the cell surface of HEK-293 cells (Fig. 8, Control). After 15 min of agonist treatment, a marked aggregation or clustering of A2AR-FLAG at the cell-surface level was observed (Fig. 8, 15 min). Moreover, when cells were incubated for 2 h with the agonist, both cell-surface clustering and a reduction in the total amount of plasma membrane receptor were observed (Fig. 8, 2 h). These results agree with the biotinylation ones (Fig. 6). Similarly, A2ΔCT2AR-FLAG showed an even distribution at the plasma membrane level when expressed in HEK-293 cells (Fig. 8, Control). Interestingly, when treated with the agonist, the cell-surface distribution and amount of A2ΔCT2AR-FLAG were not altered compared with the control cells (Fig. 8). This agrees with the previous biotinylation results (Fig. 7). Overall, these results indicate that the C-terminal domain of A2AR is essential for agonist-induced clustering and internalization and suggest that a proper attachment to the α-actinin/actin cytoskeleton may be crucial for A2AR trafficking.

**DISCUSSION**

A yeast two-hybrid approach was used to identify a novel interaction between the heptaspanning membrane adenosine A2a receptor and the actin-cross-linking protein α-actinin. The interaction was verified by pull-down experiments using GST and A2aR-GST fusion constructs and by colocalization and co-immunoprecipitation experiments in transfected HEK-293 cells. Moreover, colocalization of both proteins in rat striatal primary cultures and the ability of anti-A2aR antibodies to immunoprecipitate α-actinin from rat striatal homogenates suggest that the interaction is physiologically relevant. α-Actinin is a component of the actin cytoskeleton that plays a central role by directly cross-linking the actin molecules, so the presence of a complex involving A2aR and α-actinin suggests that α-actinin may mediate receptor association with the actin cytoskeleton. Recent studies have identified filamin A, another actin-cross-linking protein similar to α-actinin, as an intracellular binding partner for other heptaspanning membrane receptors, viz. the dopamine D2 and D3 receptors (20, 21), the calcium-sensing receptor (22), and metabotropic glutamate receptors (23). A role for these interactions in the proper membrane targeting and synaptic localization of all these receptors has been proposed. Filamin A plays a particularly important...
role in dopamine D₂ receptor plasma membrane expression (21) and cell-surface clustering (20). Similarly, one important result achieved in this work is that the attachment of A₂A R to the actin cytoskeleton through α-actinin was revealed as a prerequisite for its agonist-induced plasma membrane clustering.

Although we present here the first evidence for binding of α-actinin to a heptaspanning membrane receptor, it has already been shown to interact with other types of cell receptors such as the ATP-gated ion channel P2X₇ (24) and glutamate NMDA receptors, which are clustered and localized at postsynaptic sites by interactions with postsynaptic density protein-95 (25) and α-actinin-2 (26). An NMDA receptor and a α-actinin-2 has been reported in rat cortex and striatum (9), specially in the latter, where both α-actinin-2 mRNA and protein were shown to accumulate. Compared with other adenosine receptors, A₂A R is also specially concentrated in the striatum, where we have found a close association with α-actinin by colocalization and co-immunoprecipitation studies. It should be noted that, in rat striatal neurons, NMDA currents are inhibited by the A₂A R agonist CGS-21680. This inhibition was shown to be dependent on an intact α-actinin/actin cytoskeleton, as treatment with the actin-depolymerizing agent cytochalasin B prevents A₂A R-induced NMDA current inhibition (27). Hence, the actin cytoskeleton (and α-actinin particularly) most likely has a role in the functional regulation of NMDA action by A₂A R in rat striatum.

There is a growing body of evidence suggesting that actin plays a role in endocytosis (28, 29). Using drugs that inhibit proper actin polymerization, Lunn et al. (30) abrogated the internalization of two heptaspanning membrane receptors, viz. bombesin and endothelin receptors and the tyrosine kinase receptor for epidermal growth factor. As receptor-mediated endocytosis is a critical phenomenon in receptor down-regulation, ligand degradation, and signal termination, we were interested in the potential role of actin in agonist-dependent A₂A R internalization. In this work, a β-arrestin-dependent mechanism for A₂A R agonist-induced internalization was demonstrated. A₂A R was able to rapidly recruit β-arrestins to the cell surface upon agonist challenge, and a negative mutant of β-arrestin was shown to inhibit agonist-induced A₂A R internalization. In a previous study, we examined the effect of cytochalasin D, an agent that caps the growing end of actin filaments and thus disrupts actively turning over actin stress fibers (31), on the endocytosis of A₂A R that occurs after β-arrestin recruitment. Our results show that, whereas A₂A R can normally internalize in HEK-293 cells transiently transfected with A₂A R-FLAG, the exposure of these cells to cytochalasin D inhibits the internalization of the receptor, confirming a functional relationship between A₂A R and the underneath actin structure and suggesting that this internalization process depends on actively turning over actin stress fibers rather than on cortical actin, which remains less affected by the action of cytochalasin D (30, 31). Moreover, a mutant of A₂A R lacking the C-terminal domain, which could not interact with α-actinin in transfected HEK-293 cells, did not internalize upon agonist exposure. These results show that the C-terminal tail of A₂A R is an essential domain for its internalization; and as this domain mediates the interaction with α-actinin, they suggest that a proper attachment of the receptor to the α-actinin/actin cytoskeleton is a prerequisite for its agonist-induced β-arrestin-mediated internalization.

As recently proposed for other G protein-coupled receptors, once internalized, the interaction between receptors and β-arrestins also triggers the ERK cascade, where β-arrestins can behave as agonist-regulated molecular adapters and scaffolds (32), thus allowing activated ERKs to target specific subcellular domains (33). α-Actinin also interacts with proteins involved in signal transduction such as the MEK activator MEKK1 (34) and ERK (35). Recently, we reported that A₂A R can signal through the extracellular signal-regulated MAPK cascade (10). Taking all this evidence together, it seems that, apart from β-arrestins, α-actinin has a dual role as an actin cytoskeleton component and as a scaffolding protein, anchoring receptors to their target signaling molecules and so ensuring a rapid and efficient signal transduction. A similar hypothesis has been suggested for another actin-cross-linking protein, filamin A, as this protein interacts with MEK1/2 and p38 kinases (36) and the Ras-related GTPases Rac, RhoA, Cdc42, and Rap1 (37). So, consistent with a double function as a scaffolding and adapter protein, the interaction of filamin A increases the coupling efficiency of the dopamine D₂ receptor and adenylylate cyclase (20, 21). Moreover, α-actinin-2 competes with calmodulin for binding to the C-terminal domain of the NMDA NR1 subunit, which is involved in the calcium-dependent inactivation of the NMDA receptor (38, 39). This indicates that α-actinin-2 also plays a regulatory role in the calmodulin/NMDA receptor interaction and does not merely anchor the receptor to the actin cytoskeleton. Further studies to establish the effects of α-actinin in A₂A R activation of either adenylylate cyclase or the ERK1/2 cascade in transfected cells as well as in neuronal primary cultures will be needed to understand the role of the cytoskeleton in adenosine neuromodulation.

In summary, a direct interaction between α-actinin and A₂A R has been identified by the yeast two-hybrid system and confirmed by convergent techniques in transfected HEK-293 cells and in more physiological models such as cultured neurons and rat striatum. Finally, we have shown that agonist-mediated clustering and internalization of A₂A R are mediated by its C-terminal tail and are dependent on an intact α-actinin/actin network, thus providing a functional link between this adenosine receptor and the actin cytoskeleton.

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