Presynaptic Control of Striatal Glutamatergic Neurotransmission by Adenosine A₁–A₂ₐ Receptor Heteromers

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The functional role of heteromers of G-protein-coupled receptors is a matter of debate. In the present study, we demonstrate that heteromerization of adenosine A₁ receptors (A₁Rs) and A₂ₐ receptors (A₂ₐRs) allows adenosine to exert a fine-tuning modulation of glutamatergic neurotransmission. By means of coimmunoprecipitation, bioluminescence and time-resolved fluorescence resonance energy transfer techniques, we showed the existence of A₁R–A₂ₐR heteromers in the cell surface of cotransfected cells. Immunogold detection and coimmunoprecipitation experiments indicated that A₁R and A₂ₐR are colocalized in the same striatal glutamatergic nerve terminals. Radioligand-binding experiments in cotransfected cells and rat striatum showed that a main biochemical characteristic of the A₁R–A₂ₐR heteromer is the ability of A₂ₐR activation to reduce the affinity of the A₁R for agonists. This provides a switch mechanism by which low and high concentrations of adenosine inhibit and stimulate, respectively, glutamate release. Furthermore, it is also shown that A₁R–A₂ₐR heteromers constitute a unique target for caffeine and that chronic caffeine treatment leads to modifications in the function of the A₁R–A₂ₐR heteromer that could underlie the strong tolerance to the psychomotor effects of caffeine.

Key words: adenosine A₁ receptor; adenosine A₂ₐ receptor; heteromeric receptors; glutamate; striatum; caffeine

Introduction

Of the four known adenosine receptors (A₁, A₂ₐ, A₂ₐ, and A₃), A₁ receptor (A₁R) and A₂ₐ receptor (A₂ₐR) are primarily responsible for the central effects of adenosine (Dunwiddie and Masino, 2001). The same receptors are also the main target of nontoxic psychostimulant doses of caffeine, the most consumed psychoactive drug in the world (Fredholm et al., 1999). In addition to their postsynaptic location in different brain regions, A₁R and A₂ₐR can be found presynaptically. A₁R and A₂ₐR are coupled to G₁₂₀₅₄ and G₁₆₅₄₆ proteins, respectively (Dunwiddie and Masino, 2001). Stimulation of presynaptic A₁Rs receptors decreases the probability of neurotransmitter release, whereas activation of presynaptic A₂ₐRs enhances neurotransmitter release (Yawo and Chu-hma, 1993; Wu and Saggau, 1997; O’Kane and Stone, 1998; Lopes et al., 2002; Quarta et al., 2004b).

Previous studies have provided evidence for functional antagonistic interactions between A₁Rs and A₂ₐRs that modulate glutamate release in the striatum and hippocampus (O’Kane and Stone, 1998; Lopes et al., 2002; Quarta et al., 2004b). The coexistence of both facilitatory A₂ₐRs and inhibitory A₁Rs in the same terminal is intriguing, particularly in view of their opposite functional effects. In the present study, we use radioligand-binding, coimmunoprecipitation, bioluminescence resonance energy transfer (BRET) and time-resolved fluorescence resonance energy transfer (TR-FRET) techniques to demonstrate that A₁R and A₂ₐR form A₁R–A₂ₐR heteromers in mammalian cells and in striatal glutamatergic nerve terminals. The main biochemical characteristic of the A₁R–A₂ₐR heteromer is the ability of A₂ₐR activation to reduce the affinity of the A₁R for agonists, providing a switch mechanism by which low and high concentrations of adenosine inhibit and stimulate, respectively, glutamatergic neurotransmission. The present study is the first indication that heteromerization of different receptors for the same modulator constitutes a unique way to presynaptically regulate neurotransmission and also is the first indication that A₁R–A₂ₐR heteromers may be involved in the acute and chronic effects of caffeine.
Materials and Methods

Animals, cell culture, and transfection. Male Sprague Dawley or Wistar rats (Charles River Laboratories, Wilmington, MA) were used, where indicated. Caffeine was administered chronically by giving free access to bottles containing a solution of 1.0 mg/ml caffeine anhydride base (Sigma, St. Louis, MO) in tap water for 14 d (chronic group) as the only fluid available for drinking. HEK-293 cells were grown in DMEM (Sigma) as described previously (Ciruela et al., 2001; Canals et al., 2003). All animal experiments were performed in accordance with the National Institutes of Health and Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research (National Research Council, 2003).

Cells were transiently transfected with the DNA encoding for the proteins specified in each case by calcium phosphate precipitation. The human hemagglutinin (HA)-A2AR and Flag-A1R construct were obtained as previously described (Ciruela et al., 2001; Canals et al., 2003). These receptors were also subcloned in frame with either Renilla luciferase (A2AR-Rluc) or with the enhanced yellow florescent protein (A2AR-YFP).

Immunoprecipitation and Western blot analysis. Transiently transfected HEK cells or striatal synaptosomes were solubilized in ice-cold lysis buffer [PBS, pH 7.4, containing 1% (v/v) Nonidet P-40] for 30 min on ice. The solubilized preparation was then centrifuged at 13,000 × g for 30 min, and the supernatant (1 mg/ml) was processed for immunoprecipitation, as described previously (Ciruela et al., 2001; Canals et al., 2003). Immune complexes in sample buffer (8 x 10^4, 2% SDS, 100 mM DTT, 375 mM Tris, pH 6.8) were dissoicated by heating to 37°C for 2 h and resolved by SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membranes using a semidry transfer system and immunoblotted with the indicated primary antibody. The blots were then incubated with a secondary horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:60,000). The immunoreactive bands were developed using a chemiluminescent detection kit. The primary antibodies used were as follows: anti-Flag monoclonal antibody and anti-HA monoclonal antibody (Sigma) and rabbit anti-A2AR and anti-A1R polyclonal antibodies (Affinity BioReagents, Golden, CO). To test the specificity of the anti-A2AR polyclonal antibody in striatal tissue, a preincubation with the immunizing peptide (C309)QPQPPIPEDPEEKAED(326) (ab5893; Abcam, Cambridge, MA) was performed. This preincubation completely abrogated the staining observed in striatal tissue (data not shown).

Immunostaining. For immunohistochemistry, transiently transfected HEK cells or rat striatal synaptosomes were fixed in 4%paraformaldehyde for 15 min and washed with PBS containing 20 m osm glycerine (buffer A) to quench the aldehyde groups. Then, after permeabilization with buffer A containing 0.2% Triton X-100 for 5 min, cells or striatal synaptosomes were treated with PBS containing 1% bovine serum albumin (buffer B). After 1 h at room temperature, cells or synaptosomes were labeled with the indicated primary antibody for 1 h, washed, and stained with the indicated secondary antibody. Samples were rinsed and observed in a confocal microscope (Ciruela et al., 2001; Canals et al., 2003; Rodrigues et al., 2005). The primary antibodies used were as follows: anti-HA monoclonal antibody (2 µg/ml; Sigma), rabbit anti-Flag polyclonal antibody (2 µg/ml; Sigma), mouse anti-A2AR (2 µg/ml; Upstate Biotechnology, Lake Placid, NY), rabbit anti-A1R (1:200; Affini-ty Bioagents), guinea pig anti-vGlut1 (1:5000; Chemicon, Temecula, CA) and guinea pig anti-vGlut2 (1:5000; Chemicon). The secondary antibodies used were as follows: Alexa Fluor 488-conjugated goat anti-mouse IgG (1:200; Invitrogen, Eugene, OR), Texas Red-conjugated goat anti-rabbit IgG (1:1000; Invitrogen), Alexa Fluor 598-conjugated goat anti-guinea pig (1:200; Invitrogen), and Alexa Fluor 350-conjugated goat anti-rabbit (1:200; Invitrogen). For ultrastructural analysis, preembedding techniques for single and double labeling were used, as described previously (Lujan et al., 1996). Briefly, free-floating sections were incubated in Triton-buffered saline containing 10% of normal goat serum (TBS-NBS) for 1 h. After blocking, sections were incubated during 48 h with TBS-NBS containing mouse anti-A2AR antibody (2 µg/ml; Upstate Biotechnology) and/or rabbit-anti-A1R antibody (2 µg/ml; Affinity Bioagents). When a single primary antibody was used, it was visualized by the silver-intensified immunogold reaction. When two primary antibodies were used, one of them (anti-A2AR antibody) was visualized by the immunoperoxidase reaction and the second one (anti-A1R antibody) by the silver-intensified immunogold reaction. The following secondary antibodies were used: goat anti-rabbit coupled to 1.4 nm gold, goat anti-mouse coupled to 1.4 nm gold, and biotinylated goat anti-mouse antibody. After washing in TBS, sections were processed and observed as described previously (Lujan et al., 1996). BRET and TR-FRET. Forty-eight hours after transfection, HEK cells were rapidly washed twice in PBS, detached, and resuspended in the same buffer. The protein was determined using a Bradford assay kit (Bio-Rad, Hercules, CA). To quantify A2AR-Rluc and A1R-YFP expression, a cell suspension (20 µg of protein) was distributed in duplicate into 96-well transparent bottom microplates (Corning, Corning, NY). The fluorescence and luminescence were determined as described previously (Canals et al., 2003). For BRET measurement, 20 µg of cell suspension was distributed in duplicate in 96-well opaque microplates (Corning) and 5 mM coelenterazine H was added. After 1 min, the readings were collected by using a Fusion microplate analyzer (Packard, Meridian, CT) that allows the integration of the signals using filters with the appropriate bandpass. The BRET ratio is defined as [(emission at 510–590)/(emission 440–500)] – cf., where cf. corresponds to (emission at 510–590)/(emission at 440–500) for the A2AR-Rluc construct expressed alone in the same experiment. Time-resolved FRET was performed as described previously (McVey et al., 2001). Briefly, transiently transfected HEK cells were detached, washed twice with PBS, and incubated in PBS containing 50% fetal bovine serum and 5 mM Eu a,E Ta 3+ -labeled anti-Flag antibody (CIS bio, Ga/Yvette, France) and 20 mM albomocyanin-labeled anti-HA antibody (CIS bio) during 3 h in constant rotation at room temperature. After washing, cells were placed into a 96-well microtiter plate and analyzed using a Victor 2 (PerkinElmer, Wellesley, MA) configured for time-resolved fluorescence.

Radioligand-binding experiments. Membrane samples from rat striatum or from transfected HEK cells were obtained as described previously (Casado et al., 1990; Herrera et al., 2001). Displacement experiments were performed by incubating (120 min) membranes (0.25 mg/ml striatal protein or 0.19 mg/ml HEK cells) at 25°C in 50 mM Tris-HCl buffer, pH 7.4, containing 10 mM MgCl2 and 2 mM adenosine deaminase (EC 3.5.4.4; Roche, Basel, Switzerland) with the indicated concentrations of [3H]-labeled R-phenylisopropyladenosine ([3H]-R-PIA) (30.5 Ci/mmol; Moravek Biochemicals, Brea, CA) or [3H]-labeled 2-p-(2-carboxyethyl)phenylamino-5'-N-ethylcarboxamido adenosine HCl ([3H]-CGS21680) (45 Ci/mmol; PerkinElmer) in the absence or presence of increasing concentrations of nonlabeled compounds (Sarrio et al., 2000). Nonspecific binding was determined in the presence of 10 µM 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) (Sigma) for A1Rs or 10 µM 8-CPT-cAMP (Upstate Biotechnology, Lake Placid, NY) for A2ARs. Data were analyzed by converting the radioactivity remaining bound to the specific binding (i.e., the nonspecific binding was subtracted). Kinetic parameters were obtained by the nonlinear regression program GRAFIT. A modified F test was used to analyze whether the fit to the two-site model significantly improved on the fit to one-site model, and p < 0.05 was considered a significant improvement when no significant improvement over the one-site model was detected, the p values were >0.30.

Calcium determination. Transiently transfected HEK cells (10⁶ cells/ml) were loaded with 5 mM fura-2 AM for 30 min at 37°C. After washing the cells, calcium peak induction was achieved by the addition of 50 nM R-PIA and/or 200 nM CGS21680. Intracellular calcium was determined as described previously (Ciruela et al., 2001).

Striatal synaptosomes preparation and measurement of evoked [3H]glutamate release. Striatal synaptosomes prepared for immunocytochemical analysis were obtained through a discontinuous Percoll gradient (Ro-
The release of [3H]glutamate from rat striatal nerve terminals was performed as described previously (Lopes et al., 2002; Rodrigues et al., 2005). The synaptosomes were stimulated with 20 mM K+ (isomolar substitution of NaCl by KCl in the Krebs’ solution) at 3 and 9 min after starting sample collection (S1 and S2). Tested drugs were added 2 min before S2 onward, whereas modifiers present in S1 and S2 were added from 10 min before starting sample collection onward. Radioactivity was expressed in terms of disintegrations per second per milligram of protein (bequerels per milligram) in each chamber. The percentage of modification of the evoked release caused by the tested drugs was determined by measuring the percentage of modification of the ratio S2/S1 in comparison with the ratio S2/S1 of the respective control. Adenosine (0.1–100 μM), N7-cyclopentyladenosine (CPA) (100 nM), CGS (10 nM), DPCPX (50 nM), and 5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c](pyrimidine (SCH152681) (50 nM), when present during S1 and S2, did not cause any effect per se on the S2/S1 ratio. We further confirmed that the presence of either CPA or CGS21680 during S1 and S2 did not lead to a desensitization of the A1R-mediated or A2AR-mediated modulation of glutamate release; in fact, when applied during S1 and S2, CGS21680 (10 nM) enhanced glutamate release by 28.5 ± 1.8% during S1 and by 34.9 ± 3.6% during S2, whereas CPA (100 nM) inhibited glutamate release by 10.8 ± 0.7% during S1 and by 10.4 ± 1.0% during S2 (n = 6).

Results

A1R–A2AR heteromers in the cell surface of transfected cells

From extracts of HEK cells transiently transfected with Flag-A1Rs and HA-A2ARs, the mouse anti-Flag antibody co-immunoprecipitated a band of ~42 kDa, which corresponds to the HA-A2AR (Fig. 1a). This band did not appear in immunoprecipitates from cells only transfected with the cDNA for either HA-A2ARs or Flag-A1Rs (Fig. 1a). Conversely, in extracts from these cells, the anti-HA antibody co-immunoprecipitated a band of ~38 kDa corresponding to the Flag-A1R (Fig. 1a).

A1R–A2AR heteromerization was tested using BRET assays. A positive and saturable BRET signal for the transfer of energy between A2AR-R-Fluc and A1R-YFP was obtained (Fig. 1b) in cells cotransfected with a constant concentration of the A2AR-R-Fluc and increasing concentrations of A1R-YFP. As the pair A2AR-R-Fluc plus A1R-YFP argues for the selectivity of the interaction between A2ARs and A1Rs, and also suggest that the distance between donor (A2AR-R-Fluc) and acceptor (A1R-YFP) is ≤10 nm. To assess whether these A1R–A2AR heteromers occur at the plasma membrane level, TR-FRET was assayed in cells cotransfected with Flag-A1Rs and HA-A2ARs. Because Flag and HA tags were localized extracellularly at the N terminus of these receptors, only the A1Rs and A2ARs localized at the plasma membrane of intact cells were detected and gave a positive strong TR-FRET signal (acceptor/donor emission, mean ± SEM, 0.08 ± 0.002; n = 3) compared with a mixture of cells previously transfected with only A1Rs or A2ARs and then mixed before the assays (acceptor/donor emission, 0.011 ± 0.002; n = 3). Confocal microscopy analysis of nonpermeabilized HEK cells transfected with the cDNAs for Flag-A1Rs and HA-A2ARs confirmed an overlapped distribution of the two proteins on the cell surface (Fig. 1c).

Biochemical characteristics of the A1R–A2AR heteromers

Radioligand-binding experiments using the A1R agonist [3H]R-PIA and the A2AR agonist [3H]CGS21680 were performed in A1R–A2AR cotransfected cells and cells transfected with either A1Rs or A2ARs (Table 1). Kp values for the binding of R-PIA to A1R and for the binding of CGS21680 to A2AR were similar in single- or double-transfected cells (Table 1), indicating that heteromerization does not modify the affinity of the A1Rs or A2ARs for agonists. A1R showed the same Kp for caffeine in A1R as in A1R–A2AR cotransfected cells (Table 1). In single-transfected cells,
A2ARs displayed higher affinity for caffeine than A1Rs (Table 1). Interestingly, the affinity of A2ARs for caffeine in A1R–A2AR-cotransfected cells was 12-fold lower to that compared with A2AR-transfected cells (p < 0.001) (Table 1). The affinity of the A2AR for CGS21680 and caffeine was not altered when cotransfected with the dopamine D2 receptor (D2R), although they form A1R–D2R heterodimers (Canals et al., 2003) (Table 1).

In cells cotransfected with both A1Rs and A2ARs, but not in cells expressing only A1Rs, the affinity of A1Rs for[^3H]R-PIA decreased in the presence of the A2AR agonist CGS21680, indicating that A2AR activation reduces the affinity of A1R for its agonist. As illustrated in Figure 2a, the displacement of[^3H]R-PIA by CGS21680 in membranes from A1R–A2AR-cotransfected cells was significantly (p < 0.01) better fitted by a two-site model than by a single-site model (Fig. 2a, solid vs dashed line), whereas it was better fitted (p < 0.01) to a single-site model rather than a two-site model in membranes from A1R single-transfected cells (Fig. 2b, solid vs dashed line). At low concentrations of CGS21680, when it binds preferentially to A2ARs (at concentrations of CGS21680 < 500 nM), the direct binding of CGS21680 to A1Rs is <1%, according to the calculated affinity of A1R for CGS21680, CGS21680 significantly (p < 0.05) decreased the binding of[^3H]R-PIA to the A1R from an EC_{50} value of 3.0 ± 0.4 μM to 30 ± 10 nM (means ± SEM). The EC_{50} value corresponds to one-half of the maximum inhibitory effect of CGS21680 on the binding of[^3H]R-PIA to the A1R (or the high-affinity site calculated using the two-site model). In contrast, the displacement of[^3H]CGS21680 by R-PIA was not affected in the membranes from cells cotransfected with A1Rs and A2ARs (data not shown). This means that the binding characteristics of A2ARs are preserved in the A1R–A2AR heterodimer, whereas the binding characteristics of A1Rs become controlled by A2ARs in this heterodimer.

The ability of A2ARs to decrease A1R-mediated effects after formation of the A1R–A2AR heterodimer was confirmed in experiments measuring intracellular calcium levels in HEK cells transiently expressing Flag-A2ARs plus HA-A1ARs. The intracellular calcium peak induced by the A1R agonist, R-PIA (50 nM), was similar in HEK cells transfected with A1R alone or A1R plus A2AR (Fig. 2c) (95 ± 15 and 86 ± 12 nM, respectively). However, pre-incubation with 50 nM CGS21680, which was by itself ineffective, significantly reduced the calcium peak obtained in response to A1R activation in cells doubly transfected with both receptors (Fig. 2c) to 41 ± 16 nM. This contrasted with the inability of CGS21680 (50 nM) to affect A1R-mediated recruitment of intracellular calcium in cells expressing only Flag-A1Rs. R-PIA (50 nM) or CGS21680 (50 nM) failed to modify intracellular calcium levels in nontransfected HEK-293 cells (Fig. 2c).

A1R–A2AR heteromers in rat striatum

Immunoelectron microscopy, using preembedding immunogold techniques, revealed the presence of A2ARs immunoreactivity along the extrasynaptic plasma membrane of axon terminals establishing asymmetric synapses (Fig. 3a), as well as in the presynaptic active zone of asymmetric synapses facing dendritic shafts and spines (Fig. 3b). A2ARs were also observed in postsynaptic structures along the extrasynaptic and perisynaptic plasma membrane of dendritic shafts and spines, establishing asymmetrical synapses with axon terminals (Fig. 3a–c). Similarly, immunoreactivity for the A1R was localized along the extrasynaptic plasma membrane and presynaptic active zone of axon terminals in asymmetric synapses and along the extrasynaptic plasma membrane of dendritic shafts and spines (Fig. 3d,e) establishing asymmetrical synapses with axon terminals. Interestingly, both receptors colocalized in both presynaptic and postsynaptic structures of asymmetrical, putative glutamatergic, synapses (Fig. 3f–h). This is the first direct anatomic evidence for adenosine A2ARs and A1Rs colocalization in the same neuronal compartments.

To test the presence of A1R–A2AR heteromers in the rat striatum, competitive-inhibition experiments by CGS21680 of[^3H]R-PIA binding were also performed in membrane preparations from rat striatum. As observed in cells cotransfected with A1Rs and A2ARs, the obtained displacement data in rat striatal membranes were significantly (p < 0.001) better fitted by a two-site model than by a single-site model (Fig. 4a). The IC_{50} value, which reflects the binding of CGS21680 directly to the A1R (low-affinity site calculated using the two-site model), and the EC_{50} value, which refers to the effect of CGS21680 on the binding of R-PIA to A1R (high-affinity site calculated using the two-site model) were 1.8 ± 0.5 μM and 22 ± 6 nM, respectively (means ± SEM). These values were strikingly similar to those obtained using A1R–A2AR-cotransfected cells (3.0 ± 0.4 μM and 30 ± 10 nM, respectively). The activation of A1R with R-PIA did not modify the affinity of A2ARs for its agonist CGS21680 in rat striatal membranes (data not shown), as observed in cells cotransfected with A1Rs and A2ARs. Another correlate of the existence of A1R–A2AR heteromers in the striatum, based on the findings obtained in cells cotransfected with A1Rs and A2ARs, was that the affinity of rat striatal A2ARs for caffeine was four times lower than the affinity of A1Rs for caffeine (see below).

A1R–A2AR heteromeric complexes in striatal glutamatergic nerve terminals

The preferential colocalization of A1R and A2AR in glutamatergic terminals was confirmed by immunocytochemistry and coinmunoprecipitation. A1R and A2AR immunoreactivity could be
A1R–A2AR heteromers in striatal glutamatergic nerve terminals. Overall, these results agree with the occurrence of functional A1Rs and A2ARs, whereas only 1.5 ± 0.9% are endowed with A2ARs but not with A1Rs and 9 ± 1% with A1Rs but not with A2ARs (Fig. 5b). The number of glutamatergic terminals that do not express any of the receptors is <30%. As shown in Figure 5c, an antibody against A2ARs was able to immunoprecipitate A1Rs from solubilized rat striatal nerve terminals. These results demonstrate that A1Rs and A2ARs can form heteromeric receptor complexes in the striatal nerve terminals.

To prove a direct effect of presynaptic A1Rs on glutamate release, the evoked release of glutamate was analyzed from superfused rat striatal nerve terminals loaded with [3H]glutamate. The A1R agonist CPA (100 nM) inhibited (mean ± SEM; 31.6 ± 2.6%) the K+ evoked [3H]glutamate release and this effect was prevented by preincubation with the selective A1R antagonist DPCPX (50 nM) (Fig. 6a), which did not have any significant effect on its own (data not shown). The inhibitory effect of CPA was also prevented when synaptosomes were preincubated with the A2AR agonist CGS21680 (10 nM) (Fig. 6a), showing the A2AR-mediated negative modulation of A1R function, as occurred in HEK cells. In contrast, CGS21680 facilitated (57.7 ± 6.4%; n = 3) the K+ evoked [3H]glutamate release, and this effect was prevented by the selective A2AR agonist SCH58261 (50 nM) but was not modified when synaptosomes were preincubated with CPA (Fig. 6a). SCH58261 alone did not have any effect on glutamate release (data not shown). When CGS21680 (10 nM) and CPA (100 nM) were simultaneously added, a facilitatory effect was obtained (44.5 ± 8.6%; n = 6), which was significantly (p < 0.05) higher than the arithmetic sum of the separate effects of 10 nM CGS21680 and 100 nM CPA (i.e., if these effects were purely additive), as illustrated by the dashed bar in Figure 6a. Overall, these results agree with the occurrence of functional A1R–A2AR heteromers in striatal glutamatergic nerve terminals testified by the ability of A2ARs to abrogate A1R-mediated effects.

Finally, the addition of increasing concentrations of adenosine (0.1–100 μM) demonstrated its ability to biphasically modulate the evoked release of glutamate from striatal nerve terminals. At a concentration of 0.1 μM, adenosine did not significantly modify glutamate release (n = 6); at 1 μM, adenosine significantly decreased (23.6 ± 4.7%; n = 8; p < 0.05), whereas, at 10 and 100 μM, it significantly facilitated K+ evoked [3H]glutamate (30.4 ± 7.5 and 17.6 ± 3.5%; n = 6; p < 0.05 in both cases).

Figure 3. A1R–A2AR colocalization in rat striatum. a–c, Immunogold particles for the A2ARs were observed at the presynaptic level along the extrasynaptic plasma membrane (arrows) of axon terminal (B), as well as in the presynaptic active zone (crossed arrows), facing dendritic shafts (D) and spines. A1Rs were also observed at the postsynaptic level along the extrasynaptic and perisynaptic plasma membrane of dendritic shafts (D) and spines (arrowheads), establishing asymmetrical synapses with axon terminals. d, e, Similarly to A2ARs, immunogold particles for the A1R were localized along the presynaptic active zone of axon terminals (B; crossed arrows) and along the extrasynaptic plasma membrane of dendritic shafts (D) and spines (arrowheads) establishing asymmetrical synapses with axon terminals. f, g, Presynaptic colocalization of A1Rs and A2ARs. Peroxidase reaction product (immunoreactivity for A1Rs) filled axon terminals (B) establishing asymmetrical synapses with dendritic shafts (D) or spines (S), in which immunoparticles (immunoreactivity for A2ARs) were localized along the extrasympaptic plasma membrane (arrow). h, Postsynaptic colocalization of A1Rs and A2ARs. Peroxidase reaction product (immunoreactivity for A1Rs) filled dendritic shafts (D) establishing asymmetrical synapses with axon terminals (B), in which immunoparticles (immunoreactivity for A2ARs) were localized along the synaptic plasma membrane (arrowhead). Scale bar, 0.2 μm.

Figure 4. Intramembrane A1R–A2AR interaction in rat striatum. Effect of chronic caffeine treatment. Modulation of [3H]R-PIA binding to A1Rs by A2AR activation. Striatal membranes (0.25 mg/ml of control (a) or caffeine-treated rats (b) were incubated with 0.9 nM [3H]R-PIA in the absence or presence of increasing concentrations of CGS21680 in 50 mM Tris-HCl containing 10 mM MgCl2 and 2 U/ml adenosine deaminase. Binding experiments were performed as described in Materials and Methods. The data were adjusted to one single binding site (dashed line) or to two binding sites (solid line).

A1R–A2AR intramembrane interaction and caffeine tolerance. An unresolved issue about caffeine is the strong tolerance for many of its behavioral and biochemical effects that develop after chronic treatment (Holtzman and Finn, 1988; Jacobson et al.,
1996; Fredholm et al., 1999). To test a possible role of A1R–A2AR heteromers in this phenomenon, competitive-inhibition experiments of \( [3H] \)R-PIA binding using CGS21680 were performed in striatal membrane preparations of caffeine-treated rats (1 mg/ml in the drinking water for 14 d; average caffeine consumption, mean ± SEM, 120 ± 4 mg · kg\(^{-1} \) · d\(^{-1} \)), and the results compared with those obtained in naive animals. Again, the displacement curve of \( [3H] \)R-PIA binding by CGS21680 in striatal membranes from caffeine-treated rats was significantly (\( p < 0.001 \)) better fitted by a two-site model than by a single-site model (Fig. 4b). The \( IC_{50} \) value (mean ± SEM) corresponding to the binding of CGS21680 to A1R (1.7 ± 0.5 \( \mu M \)) was similar to that obtained in naive rats (1.8 ± 0.5 \( \mu M \)) as well as the \( [3H] \)R-PIA binding to these striatal membranes when no displacer was present (Fig. 4). However, the inhibition of the \( [3H] \)R-PIA binding to A2AR when CGS21680 binds to the A2AR displayed an \( IC_{50} \) value about threefold lower than the value obtained in naive rats (mean ± SEM, 8 ± 3 and 22 ± 6 \( nM \), respectively). These results indicate that caffeine pretreatment alters the function of the A1R–A2AR heteromers, increasing significantly (\( p < 0.02 \)) the potency of an A2A agonist to modulate A1Rs. The agonist binding characteristics for both striatal A1R and A2AR were not modified by caffeine treatment. Thus, in competition experiments using \( [3H] \)R-PIA, \( K_{D} \) values (means ± SEM) for the binding of R-PIA to A1R in control and caffeine-treated animals were 0.1 ± 0.05 and 0.13 ± 0.06 \( nM \), respectively. In competition experiments using

Figure 5. Adenosine receptors in glutamatergic striatal nerve terminals. a, Immunocytochemical identification of A2AR (green) and A1R (blue) in the glutamatergic population of rat striatal nerve terminals (identified as vGluT-1 and vGluT-2 immunoreactive; red). In the superimposed picture of this triple-immunocytochemical labeling (merge) of this representative field, the arrows indicate the A1R/A2AR/vGluT-containing nerve terminals. b, The quantification of images of three different fields per coverslip from four experiments using different synaptosomal preparations from different animals confirmed the predominant colocalization of A1Rs and A2ARs in rat striatal glutamatergic terminals (results are means ± SEM). c, Coimmunoprecipitation of A1R and A2AR from rat striatal synapses. Solubilized synaptosomes were immunoprecipitated using rabbit anti-A1R polyclonal antibody (5 \( \mu g \)) (lane 1), irrelevant rabbit polyclonal antibody (5 \( \mu g \)) (lane 2), and mouse anti-A2AR monoclonal antibody (2 \( \mu g \)) (lane 3). Solubilized membranes (Crude) and immunoprecipitates (lanes 1–3) were analyzed by SDS-PAGE and immunoblotted using rabbit rabbit anti-A1R antibody (1:1000) and HRP-conjugated swine anti-rabbit IgG as a secondary antibody. IB, Immunoblot.

Figure 6. Effect of A1R and A2AR activation on evoked release of glutamate from striatal nerve terminals. Superfused synaptosomes, previously loaded with \( [3H] \)glutamate, were chemically (20 mM K\(^+\) for 30 s) stimulated twice (S1 and S2). a, CPA (100 nM) and CGS21680 (10 nM), present in S2, inhibited and facilitated, respectively, the evoked release of glutamate in a manner prevented by the A1R and A2AR antagonists DPCPX (50 nM) and SCH58261 (50 nM), respectively (present in S1 and S2). The inhibitory effect of CPA (100 nM) was also abolished when CGS21680 (10 nM) was present during S1 and S2, whereas the facilitatory effect of CGS21680 was not modified when CPA (100 nM) was present in S1 and S2. Coapplication of CPA (100 nM) and CGS (10 nM) in S2 facilitated the evoked release of glutamate. The dashed bar illustrates the arithmetic sum of the effects of 10 nM CGS21680 and 100 nM CPA, if these effects were purely additive. The results are means ± SEM of three to seven experiments. * \( p < 0.05 \) versus 0%; ** \( p < 0.05 \) between bars. b, Increasing concentrations of adenosine (0.1–100 \( \mu M \)) produced a biphasic effect, with low and high concentrations inhibiting and stimulating the evoked release of glutamate, respectively. The results are means ± SEM of six to eight experiments. * \( p < 0.05 \) versus 0%.
and caffeine-treated animals (Svenningsson et al., 1999), or a preferential affinity of A1R for A2AR would block A1R-mediated function, and the overall result will be a facilitation of the evoked release of glutamate (supplemental figure, available at www.jneurosci.org as supplemental material).

Under basal conditions, the relatively low extracellular levels of adenosine preferentially stimulate A1Rs, which show a higher affinity for adenosine than A2ARs (Fredholm et al., 2001). The preferential A1R stimulation in the A1R–A2AR heteromer inhibits glutamatergic neurotransmission. Under conditions of stronger adenosine release, A1R receptor activation in the A1R–A2AR heteromer would block A2AR-mediated function, and the overall result will be a facilitation of the evoked release of glutamate (supplemental figure, available at www.jneurosci.org as supplemental material).

In conclusion, we have demonstrated that A1Rs and A2ARs can form heteromers with other G-protein receptors, such as dopamine, glutamate, and ATP receptors (Agnati et al., 2003). However, the existence of heteromers constituted by different adenosine receptor subtypes had not been reported. In the present study, a precise colocalization of A1Rs and A2ARs at the presynaptic level of striatal glutamatergic synapses was demonstrated by ultrastructural analysis. The results of immunocytochemistry and coimmunoprecipitation experiments showed that the striatal A1R–A2AR heteromer is preferentially localized in the striatal glutamatergic terminals, and the experiments of evoked glutamate release directly demonstrated the ability of presynaptic A2ARs to control the A1R-mediated modulation of striatal glutamate release. Radioligand-binding experiments demonstrated that a main biochemical characteristic of the A1R–A2AR heteromer is the ability of A2AR activation to reduce the affinity of the A1R for agonists. Together, the present results indicate that the A1R–A2AR heteromer plays a role in the adenosine-mediated fine-tuning modulation of striatal glutamatergic neurotransmission.

Under basal conditions, the relatively low extracellular levels of adenosine preferentially stimulate A1Rs, which show a higher affinity for adenosine than A2ARs (Fredholm et al., 2001). The preferential A1R stimulation in the A1R–A2AR heteromer inhibits glutamatergic neurotransmission. Under conditions of stronger adenosine release, A1R receptor activation in the A1R–A2AR heteromer would block A2AR-mediated function, and the overall result will be a facilitation of the evoked release of glutamate (supplemental figure, available at www.jneurosci.org as supplemental material).

Thus, the functional characteristics of the A1R–A2AR heteromers are different from A1R or A2AR receptors and provide a rationale to understand how adenosine might facilitate or inhibit glutamatergic transmission depending on the concentration of adenosine. In fact, increasing concentrations of adenosine demonstrated a biphasic effect, with low and high concentrations of adenosine inhibiting and stimulating glutamate release from striatal nerve terminals, respectively.

The present study also provides new information about the main pharmacological targets of caffeine, A1Rs and A2ARs. To our knowledge, this is the first study that analyzes simultaneously the affinity of A1Rs and A2ARs for caffeine in transfected cells and brain (striatal) tissue. Previous studies using cloned transfected receptors showed that A2AR displays higher affinity for caffeine than A1R (Fredholm et al., 1999, 2001). In contrast, the results obtained from brain tissue are more variable, showing either no difference (Muller et al., 1997), a preferential affinity of A2AR (Svenningsson et al., 1999), or a preferential affinity of A1R for caffeine (Antoniou et al., 2005). Significant differences in the affinity of A2AR for caffeine were observed depending on its selective association with A1R. In agreement with previous studies (Fredholm et al., 2001), A2AR displays higher affinity for caffeine than A1R when studied in single-transfected cells. However, A1R–A2AR, but not A2AR–D2R, heteromerization was associated with a prominent decrease in the affinity of A2AR for caffeine, with a K_D value comparable with that observed in striatal tissue. Taking also into account that the “biochemical fingerprint” of the A1R–A2AR heteromer (the ability of A2AR activation to reduce the affinity of the A1R for agonists) could be demonstrated in striatal membrane preparations, the present results indicate that a significant amount of striatal A2AR receptors are forming heteromers with A1Rs. However, although most A1Rs and A2ARs localized in the glutamatergic terminals are forming part of A1R–A2AR heteromers, in the striatum caffeine can also target A1Rs not interacting with A2ARs localized in the dendritic spine of GABAergic enkephalinergic neurons (Rosin et al., 2003), where they can form heteromers with dopamine D2R or metabotropic mGluR receptors (Ferré et al., 2002; Agnati et al., 2003; Canals et al., 2003). In fact, A1R in the A2AR–D2R heteromer has a higher affinity for caffeine than the A2AR in the A1R–A2AR heteromer (Table 1). The blockade of these postsynaptic A2ARs could explain the decrease in the striatal expression of immediate-early genes and preproenkephalin mRNA and increased phosphorylation of DARPP-32 (dopamine- and cAMP-regulated phosphoprotein 32) (at threonine 75) described after acute caffeine treatments (Svenningsson et al., 1995; Lindskog et al., 2002).

The functional characteristics of the A1R–A2AR heteromers also allow understanding the different effects operated under acute and chronic caffeine administration. Because at relatively low adenosine levels there is little occupancy of A2ARs, most of the behavioral and biochemical effects after an acute administration of caffeine are most probably attributable to A1R blockade (Snyder et al., 1981; Solinas et al., 2002, 2005; Karcz-Kubicha et al., 2003; Quarta et al., 2004a,b; Antoniou et al., 2005). In fact, the acute systemic or intrastratial administration of caffeine enhances striatal glutamate release (Solinas et al., 2002; Quarta et al., 2004a,b). A significant finding of the present study was the increased antagonistic interaction between A1R and A2AR in the A1R–A2AR heteromer with chronic caffeine treatment. But, apart from the affinity of the A1R and A2AR for adenosine and caffeine and the A1R–A2AR intramembrane interaction, one more variable plays a substantial role when analyzing the effects taking place after chronic caffeine administration. Chronic treatment with the methylxanthine leads to an increase in the plasma and extracellular levels of adenosine (Conlay et al., 1997; Andrenes et al., 1999). The same treatment with caffeine (1 mg/ml in the drinking water for 14 d) used in the present study was reported to produce a 10-fold increase in plasma adenosine levels (Conlay et al., 1997). Under chronic caffeine treatment (or other conditions leading to increased adenosine levels), adenosine also binds and activates A1Rs, which, in addition, has a reduced affinity for caffeine. This likely scenario would lead to a situation in which the increased levels of adenosine acting on A2AR would potently inhibit A1R function by means of the increased A1R–A2AR intramembrane interaction. Under these conditions, caffeine would have little effect on A1Rs, which would already be inhibited as a consequence of the increased A1R–A2AR intramembrane intermolecular interaction. In fact, recent studies show a predominant A2AR antagonist behavioral and biochemical profile of caffeine under chronic administration with tolerance to its A1R antagonistic effects (Karcz-Kubicha et al., 2003; Quarta et al., 2004a). The present results suggest that an increased A1R–A2AR interaction in the A1R–A2AR heteromer is involved in the tolerance to the psychostimulant effects of caffeine.

In conclusion, we have demonstrated that A1Rs and A2ARs can form heteromers in striatum where they modulate glutamatergic neurotransmission and whose existence may help in comprehending the mechanisms underlying the behavioral effects pro-
duced by acute or chronic consumption of caffeine, the most consumed psychostimulant drug in the world.

References


