Allosteric Modulation of A3 Adenosine Receptors by a Series of 3-(2-Pyridinyl)isoquinoline Derivatives

ZHAN-GUO GAO, JACQUELINE E. VAN MUJULWIJK-KOEZEN, AISHE CHEN, CHRISTA E. MÜLLER, ADRIAAN P. IJZERMAN, and KENNETH A. JACOBSON

Molecular Recognition Section, Laboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland (Z.-G.G., A.C., K.A.J.); Division of Medicinal Chemistry, Leiden/Amsterdam Center for Drug Research, Leiden University, Leiden, the Netherlands (Z.-G.G., J.E.V.M.-K., A.P.I.); and Pharmaceutical Institute, University of Bonn, Bonn, Germany (C.E.M.)

Received December 19, 2000; accepted August 6, 2001

AABSTRACT

Allosteric modulators of A1 and A2A adenosine receptors have been described; however, for the A3 adenosine receptor, neither an allosteric site nor a compound with allosteric effects has been described. In this study, the allosteric modulation of human A3 adenosine receptors by a series of 3-(2-pyridinyl)isoquinoline derivatives was investigated by examining their effects on the dissociation of the agonist radioligand, [125I]I-AB-MECA, from the receptor. Several 3-(2-pyridinyl)isoquinoline derivatives, including VUF5455, VUF8502, VUF8504, and VUF8507, slowed the dissociation of the agonist radioligand [125I]-I-AB-MECA in a concentration-dependent manner, suggesting an allosteric interaction. These compounds had no effect on the dissociation of the radiolabeled antagonist [3H]PSB-11 from the A3 adenosine receptor, suggesting a selective enhancement of agonist binding. By comparison, compounds of similar structure (VUF8501, VUF8503, VUF8505), the classical adenosine receptor antagonist CGS15943 and the A1 receptor allosteric enhancer PD81723 did not significantly influence the dissociation rate of [125I]-I-AB-MECA. The effect of agonist on forskolin-induced cAMP production was significantly enhanced by VUF5455. When the subtype-selectivity of the allosteric enhancement was tested, the compounds had no effect on the dissociation of either [3H]NECA, [RF]-phenylisopropyladenosine from the A1 adenosine receptor or [3H]CGS21680 from the A2A adenosine receptor. Probing of structure-activity relationships suggested that a carbonyl group is essential for allosterism but preferred only for competitive antagonism. The presence of a 7-methyl group decreased the competitive binding affinity without a major loss of the allosteric enhancing activity, suggesting that the structural requirements for allosteric enhancement might be distinct from those for competitive antagonism.

The purine nucleoside adenosine produces numerous physiological actions via cell surface adenosine receptors. These receptors are widely distributed throughout the body and are subclassified as A1, A2A, A2B, and A3 adenosine receptors (Fredholm et al., 2000). All of the receptors belong to the G protein-coupled receptor (GPCR) family. The A3 adenosine receptor is the adenosine receptor subtype identified most recently (Zhou et al., 1992) and is involved in a variety of physiological processes (Kaiser and Quinn, 1999). Stimulation of the A3 adenosine receptor increases the release of inflammatory mediators, such as histamine, from mast cells (Hannon et al., 1995). Tumor necrosis factor-α production is inhibited at the level of transcription by the activation of the A3 receptor, which suppresses steady-state mRNA levels (Sajjadi et al., 1996). The A3 adenosine receptor is believed to be involved in ischemic preconditioning of the heart and kidney (Strickler et al., 1996; Lee and Emala, 2000). The activation of the A3 adenosine receptor is also suggested to be involved in immunosuppression (MacKenzie et al., 1994) and brain ischemia (Von Lubitz et al., 1994). Thus, adenosine receptor agonists and agents that enhance the response to adenosine would

Z.-G.G. received financial support from Gilead Sciences (Foster City, CA).

ABBREVIATIONS: GPCR, G protein-coupled receptor; I-AB-MECA, N6-(4-amino-3-iodobenzyI)-5'-N-methylcarboxamidoadenosine; R-PIA, N6-[(RF)-phenylisopropyl]adenosine; CGS21680, 2-[p-(2-carboxyethyl)phényl-ethylamino]-5'-N-ethylcarboxamidoadenosine; BCA, bicinchoninic acid; CGS15943, 5-amino-9-chloro-2-[2-furyl]-1,2,4-triazolo[1,5-c]quinazoline; CPA, N6-cyclopentyladenosine; NECA, 5'-N-ethylcarboxamidoadenosine; HEK, human embryonic kidney; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate; VUF5455, 4-methoxy-N-[7-methyl-3-(2-pyridinyl)-1-isouquinoliny]benzamide; VUF8502, 4-methyl-N-[3-(2-pyridinyl)-1-isouquinoliny]benzamide; VUF8504, 4-methoxy-N-[3-(2-pyridinyl)-1-isouquinoliny]benzamide; VUF8507, N-[3-(2-pyridinyl)-1-isouquinoliny]benzamide; VUF8501, N-[3-(2-pyridinyl)-1-isouquinoliny]benzene-carboximidamide; VUF8503, 4-methyl-N-[3-(2-pyridinyl)-1-isouquinoliny]benzene-carboximidamide; VUF8505, 4-methoxy-N-[3-(2-pyridinyl)-1-isouquinoliny]benzene-carboximidamide; PD81723, 2-amino-4,5-dimethyl-3-thienyl-[3-(trifluoromethyl)phenyl]methanone; PSB11, 8-ethyl-4-methyl-2-phenyl-(8R)-4,5,7,8-tetrahydro-1H-imidazo[2,1-I]purin-5-one.
have many clinic applications. The clinical application of adenosine analogs as directly acting, potent adenosine receptor agonists has not yet proven successful because of potential side effects. Side effects are caused by the ubiquitous presence of adenosine receptors throughout the body, which are activated by agonists indiscriminately. Thus, it is useful to consider an alternative approach that may activate adenosine receptors in a more specific manner.

Several members of the GPCR superfamily have been reported to be modulated allosterically (Birdsall et al., 1995). Allosteric modulation of GPCRs has been characterized most extensively for muscarinic receptors (Holzgrabe and Mohr, 1998), and it has been suggested that allosteric modulators may provide therapeutic advantages over orthosteric agonists. Such advantages may include greater subtype selectivity and fewer side effects (Birdsall et al., 1995; Bhattacharya and Linden, 1996; Linden, 1997). For example, diazepam and other benzodiazepines, which act as allosteric enhancers of the ion channel-coupled GABA<sub>A</sub> receptor, have acceptable side effects and are used clinically. In contrast, directly acting GABAA agonists have widespread side effects and are not used clinically. The effects of an allosteric enhancer on an organ or tissue might be event-specific because of an increase in the local concentration of the endogenous agonist. For example, hypoxic conditions increase the local production of cytoprotective adenosine. Compounds that either augment the concentration of adenosine or enhance its action locally may have a better therapeutic profile than the agonists. Additionally, neurotransmitter receptors have been reported to be less sensitive to desensitization or down-regulation by allosteric enhancers than by exogenous agonists (Birdsall et al., 1995). Thus, allosteric modulators could offer a control of receptor function not found with competitive agonists.

Within a family of receptors that bind the same endogenous ligand, the primary binding sites may be similar, because the amino acids that form this site are highly conserved (Tucek and Proska, 1995). An allosteric site, being spatially distinct from the primary site, may be located at less conserved regions on the receptor, which may provide greater subtype selectivity (Tucek and Proska, 1995; Birdsall et al., 2001).

Allosteric modulation of A<sub>1</sub> adenosine receptors was reported (Bruns and Fergus, 1990; Linden, 1997). A number of aminobenzoylthiophenes, including PD81723, were allosteric modulators of the A<sub>1</sub> adenosine receptor (Bruns and Fergus, 1990). These compounds were shown to be highly subtype-selective enhancers for A<sub>1</sub> adenosine receptors (Bruns and Fergus, 1990) and were less likely to cause desensitization and down-regulation of receptors than selective A<sub>1</sub> adenosine receptor agonists (Bhattacharya and Linden, 1996). Recently, an allosteric site on the A<sub>2A</sub> adenosine receptor was defined (Gao and IJzerman, 2000; Gao et al., 2000). However, it was not known whether there is an allosteric binding site on the A<sub>3</sub> adenosine receptor; compounds with allosteric effects at A<sub>3</sub> adenosine receptors have not been reported previously. This study demonstrated allosteric modulation of A<sub>3</sub> adenosine receptors by a number of 3-(2-pyridinyl)isoquinoline derivatives (Fig. 1, A and B).

### Experimental Procedures

**Materials.** 3-(2-Pyridinyl)isoquinoline derivatives were synthesized at Leiden/Amsterdam Center for Drug Research (Amsterdam, The Netherlands). \([^{125}\text{I}]^\text{N}-\text{(4-amin-3-iodobenzyl)}\)adenosine-5'-N-methyluronamide (\([^{125}\text{I}]\)I-AB-MECA; 2000 Ci/mmol), \([^{3}\text{H}]\)PSB-11 (53 Ci/mmol), \([^{3}\text{H}]\)N-\((\text{R})\)-phenylisopropyl)adenosine (\([^{3}\text{H}]\)R-PIA; 34 Ci/mmol), and \([^{3}\text{H}]\)CGS21680 (47 Ci/mmol) were from Amersham Pharmacia Biotech (Buckinghamshire, UK). Adenosine deaminase was obtained from Roche Molecular Biochemicals (Mannheim, Germany). Bicinchoninic acid (BCA) and BCA protein assay reagent were from Pierce Chemical (Rockford, IL). CGS15943 was a gift from Novartis (Summit, NJ). CPA and NECA were obtained from Sigma/RBI (Natick, MA). Rolipram and forskolin were purchased from Sigma (St. Louis, MO). cAMP (low pH) Immunoassay kits were from R & D Systems (Minneapolis, MN).

**Membrane Preparation.** Forebrain and striatal tissue from Wistar rats were homogenized in ice-cold 50 mM Tris-HCl buffer, pH 7.7, using an electric homogenizer. The homogenate was centrifuged at 50,000 g for 10 min at 4°C, and the pellet was washed in fresh buffer. A pretreatment with adenosine deaminase (2 units/ml) was performed. The final pellet was stored at −80°C until the binding experiments. HEK293 cells expressing human A<sub>3</sub> adenosine receptor and native RBL-2H3 mast cells with native rat A<sub>3</sub> adenosine receptors were harvested by trypsinization. Cells were centrifuged at 500 g for 10 min, and the pellet was suspended in 50 mM Tris-HCl buffer, pH 8.0, containing 10 mM MgCl<sub>2</sub>, 1 mM EDTA and 0.1 mg/ml CHAPS. The suspension was homogenized with an electric homogenizer for 5 s, and was then recentrifuged at 18,000 g for 30 min at 4°C. The resultant pellets were resuspended in buffer in the presence of 2 U/ml adenosine deaminase, and the suspension was stored at −80°C. Protein concentrations were measured with the BCA method (Smith et al., 1985).

**Competitive Binding Assays.** Binding of 0.15 nM \([^{125}\text{I}]\)I-AB-MECA (Olah et al., 1994) to membranes of HEK293 cells expressing human A<sub>3</sub> adenosine receptors (25 μg of protein) was carried out in duplicate at 37°C for 1 h in 100 μl of 50 mM Tris-HCl buffer, pH 8.0, containing 10 mM MgCl<sub>2</sub>, 1 mM EDTA, and 0.1 μg/ml CHAPS. Nonspecific binding was defined as binding in the presence of 30 μM NECA. Tested compounds were dissolved in dimethyl sulfoxide. Control incubations also contained the same concentrations of dimethyl sulfoxide.

![Fig. 1. Chemical structures of the compounds tested in this study. A, isquinoline derivatives found to enhance A<sub>3</sub> agonist binding. B, isquino-line derivatives that did not enhance A<sub>3</sub> agonist binding. C, A<sub>3</sub> allosteric enhancer.](image)
sulfoxide (≤ 1%). The reaction was terminated by filtration through GF/B filters on a cell harvester (Brandel, Gaithersburg, MD).

Disassociation of the Radiolabeled Adenosine Receptor Agonist, [125I]I-AB-MECA, from Human and Rat A3 Adenosine Receptors. [125I]I-AB-MECA (0.15 nM) was preincubated with the A3 adenosine receptor membranes at 37°C for 1 h. Nonspecific binding was determined in parallel by addition of 30 μM NECA before the preincubation. Dissociation was then initiated by the addition of NECA (final concentration, 30 μM) and test agents. Nonspecific binding in the presence of tested agents was also determined in parallel. After an additional 1.5 h at 37°C, the dissociation was terminated by filtration through GF/B filters on a cell harvester.

Dissociation of the Radiolabeled, Selective Antagonist [3H]PSB-11 from A3 Adenosine Receptors. Membranes (60 μg) were preincubated with 4 nM [3H]PSB-11 (selective A3 receptor antagonist; Müller, 2001) in a total assay volume of 100 μl for 120 min. Dissociation was initiated by addition of 30 μM NECA with or without test compounds. The time course of dissociation was measured by rapid filtration at appropriate time intervals. Nonspecific binding was measured after a 120-min incubation in the presence of 30 μM NECA.

Dissociation of [3H]R-PIA from A1 Adenosine Receptors. Binding of 1 nM [3H]R-PIA to A1 adenosine receptors in rat forebrain membranes was carried out at 37°C for 90 min in 50 mM Tris-HCl buffer, pH 7.7, with a total assay volume of 400 μl. Dissociation was initiated by the addition of 10 μM CPA with or without test compounds. Nonspecific binding was determined using 10 μM CPA. Samples were filtered after incubation at 37°C at the various time points.

Dissociation of [3H]CGS21680 from A2A Adenosine Receptors. Rat striatal membranes (80 μg) were incubated with 15 nM [3H]CGS21680 at 25°C for 90 min in 400 μl of 50 mM Tris-HCl, pH 7.7, containing 10 mM MgCl2. NECA (10 μM) was used to define nonspecific binding. Dissociation was initiated by the addition of 10 μM NECA in the presence or absence of tested compounds.

Measurement of cAMP Level. cAMP production was measured by using a commercially available, low-pH cAMP Immunomassay Kit (R&D Systems, Inc.). Briefly, HEK293 cells expressing recombinant human A3 adenosine receptors were grown to 70% confluence in 12-well plates and then treated with test compounds. Thirty minutes after the treatment, 10 μM forskolin was added to the culture medium to stimulate cAMP levels, and was incubated for an additional 15 min at 37°C. Reaction was terminated by the addition of 1 ml of 0.1 N HCl, and the cellular debris was removed by centrifugation for 5 min at 10,000g. All experiments were performed in the presence of 10 μM rolipram and 3 U/ml adenosine deaminase. cAMP levels were measured using a Bio-kineatcs reader (Bio-Tek instruments Inc., Winooski, VT).

Data Analysis. Binding experiment parameters were estimated using Prism software (GraphPad, San Diego, CA). IC50 values obtained from competition curves were converted to Kd values by using the Cheng-Prusoff equation.

### Results

Effects of the Test Compounds on the Dissociation of [125I]I-AB-MECA and [3H]PSB-11 from Human A3 Adenosine Receptors Expressed in HEK293 Cells. The dissociation of [125I]I-AB-MECA from A3 adenosine receptors following the addition of 30 μM NECA (Fig. 2) was characterized in the absence or presence of the test compounds. The dissociation rate (k-1) in the absence of the test compounds was 0.042 ± 0.005 min⁻¹, and the t1/2 of dissociation was 16.4 ± 2.5 min. The k-1 values and the half-life in the presence of 3 μM (k-1 = 0.031 ± 0.003 min⁻¹, t1/2 = 23.2 ± 3.4 min) and 10 μM (k-1 = 0.024 ± 0.003 min⁻¹, half-life = 28.5 ± 4.3 min) VUF5455 (Fig. 1A) were significantly different from those of the control values (p < 0.05). Compounds VUF8502, VUF8504 and VUF8507 also significantly decreased the dissociation at 10 μM (Table 1). The potencies of these compounds, as determined by their ability to influence the dissociation rate, were very similar. By contrast, the quinoline derivatives VUF8501, VUF8503, and VUF8505 (Fig. 1B), the A1 allosteric enhancer PD81723 (Fig. 1C) and the classical nonselective adenosine receptor antagonist CGS15943 did not influence the dissociation rate significantly (Table 1), even at 30 μM (data not shown). The k-1 values in the presence or absence of the test compounds (10 μM) are listed in Table 1. The kinetics of [125I]I-AB-MECA association to the A3 receptor was also determined, and the association rate constant k1 was calculated to be 0.061 ± 0.022 min⁻¹ nM⁻¹. The kinetic Kd value calculated by using k1 and k-1 was 0.69 ± 0.23 nM.

The dissociation rate of the novel A3 selective antagonist [3H]PSB-11 from A3 adenosine receptors expressed in HEK293 cells was either unaffected or slightly increased by these compounds (Fig. 3), suggesting that slowing of the dissociation was specific for the agonist state of the A3 adenosine receptor.

Concentration-Response Relationship for the Slowing of [125I]I-AB-MECA Dissociation from Human A3 Adenosine Receptors by Allosteric Enhancers. Fig. 4 shows the influence of increasing concentrations of the test compounds on the dissociation of [125I]I-AB-MECA in the presence of 30 μM NECA. Dissociation was allowed to proceed for 60 min before the reaction was terminated by filtration. Low concentrations of VUF5455 (<1 μM) did not modify the dissociation of [125I]I-AB-MECA. However, at concentrations > 1 μM, the dissociation rate decreased significantly. Because of the limited solubility of these compounds, the highest concentration used was 30 μM. It seemed that higher concentrations of these compounds produced more pronounced effects.

![Fig. 2. Effects of VUF5455 on the dissociation of [125I]I-AB-MECA from human A3 adenosine receptors expressed in HEK293 cells. [125I]I-AB-MECA (0.15 nM) was preassociated with the A3 adenosine receptor membranes for 1 h at 37°C in a total assay volume of 100 μl. Nonspecific binding was determined in parallel by addition of 30 μM NECA before the preassociation. After 1 h preassociation, dissociation was initiated by the addition of 30 μM NECA and was allowed to proceed for 60 min before the reaction was terminated by filtration. The IC50 values of VUF5455 are shown as half-maximal inhibitory concentrations.](image-url)
TABLE 1
The $K_i$ values of tested compounds for $^{[125]I}$I-AB-MECA (0.15 nM) binding to human A3 adenosine receptors and the dissociation rates ($k_{-1}$) in the absence and presence of the tested compounds (10 μM). The assay was carried out as described under Experimental Procedures. Data shown are the mean ± S.E. from three independent experiments performed in duplicate.

<table>
<thead>
<tr>
<th></th>
<th>$K_i$ (nM)</th>
<th>$k_{-1}$ (min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>739 ± 146</td>
<td>0.042 ± 0.007</td>
</tr>
<tr>
<td>VUF8501</td>
<td>96 ± 26</td>
<td>0.024 ± 0.003*</td>
</tr>
<tr>
<td>VUF8502</td>
<td>581 ± 82</td>
<td>0.041 ± 0.011</td>
</tr>
<tr>
<td>VUF8504</td>
<td>17.3 ± 1.7</td>
<td>0.022 ± 0.004*</td>
</tr>
<tr>
<td>VUF8505</td>
<td>307 ± 99</td>
<td>0.044 ± 0.012</td>
</tr>
<tr>
<td>VUF8507</td>
<td>204 ± 42</td>
<td>0.021 ± 0.002*</td>
</tr>
<tr>
<td>VUF5455</td>
<td>1680 ± 620</td>
<td>0.024 ± 0.003*</td>
</tr>
<tr>
<td>PD81723</td>
<td>N.D.</td>
<td>0.046 ± 0.009</td>
</tr>
<tr>
<td>CGS15943</td>
<td>185 ± 37</td>
<td>0.045 ± 0.005</td>
</tr>
</tbody>
</table>

N.D., not determined. *p < 0.05 compared with control (Student’s t test).

Competition of Test Compounds with $^{[125]I}$I-AB-MECA Binding for Human A3 Adenosine Receptors Expressed in HEK293 Cells. Fig. 5 shows the competition of $^{[125]I}$I-AB-MECA binding to A3 adenosine receptors expressed in HEK293 cells by the 3-(2-pyridinyl)isoquinoline derivatives VUF5455 and VUF8504. The $K_i$ values of various compounds for $^{125}$I-I-AB-MECA binding are listed in Table 1. VUF5455 and VUF8504, compounds with similar allosteric potencies, had the lowest and highest affinities for human A3 adenosine receptors, respectively.

Subtype-Selectivity of the Test Compounds as Allosteric Enhancers for A3 Adenosine Receptors. It was demonstrated above that these compounds decreased the dissociation rate of $^{[125]I}$I-AB-MECA from A3 adenosine receptors. An important issue was whether these compounds were selective for A3 adenosine receptors. To address this issue, we used $[^3]$HIR-PIA to investigate whether agonist dissociation from A3 adenosine receptors was altered by these modulators. VUF5455 (30 μM) had no effect on the dissociation rate of $[^3]$HIR-PIA from A1 adenosine receptors (Fig. 6). The enhancers also had no effect on the time course for the dissociation of $[^3]$HCGS21680 from A3A adenosine receptors (Fig. 7). These results suggest that the 3-(2-pyridinyl)isoquinoline derivatives are selective allosteric modulators for A3 adenosine receptors.

Structure-Activity Relationships for the Enhancement of A3 Adenosine Receptor Binding by 3-(2-Pyridinyl)isoquinoline Derivatives. All of the test compounds that displayed allosteric effects contained a carbonyl group. The replacement of the carbonyl group with an imino group resulted in a loss of the allosteric property of these compounds. The allosteric potencies of the 3-(2-pyridinyl)iso-
placed the binding of [125I]I-AB-MECA to A3 adenosine receptors, the order of potencies by which these compounds displaced the binding of [125I]I-AB-MECA was of the same order of magnitude. By comparison, the order of potencies by which these compounds displaced the binding of [125I]I-AB-MECA to A3 adenosine receptors was: VUF8504 > VUF8502 > CGS15943 > VUF8507 > VUF8505 > VUF8503 > VUF8501 > VUF5455. The compound VUF8504 was about 2 orders of magnitude more potent than VUF5455 with respect to competitive binding.

Both the allosteric and competitive potencies of these compounds for rat A3 adenosine receptors were tested. As shown in Fig. 8, the effect of VUF5455 and VUF8504 on the dissociation of [125I]I-AB-MECA from rat A3 adenosine receptors was only slightly decreased or similar to that of human A3 adenosine receptors. In contrast, their competitive binding affinities were approximately 8- and 240-fold less than at human A3 receptors, for VUF5455 (Kd = 12.8 ± 3.1 nM) and VUF8504 (Kd = 4.2 ± 0.8 nM), respectively. This demonstrated that binding enhancement could be separated from competitive antagonism.

Enhancement of the A3 Adenosine Receptor Function by VUF5455. Direct information regarding the allosteric modulation of A3 adenosine receptors was obtained from dissociation kinetic experiments. It was important to correlate data from in vitro assays with those estimated from functional experiments. Hence, we conducted a cAMP assay to observe the effects of the allosteric modulator on human A3 adenosine receptor function. The compound VUF5455 was selected for the cAMP assay, because its competitive binding to A3 adenosine receptors was relatively weak compared with other analogs. VUF5455 significantly enhanced the effect of Cl-IB-MECA on forskolin-induced cAMP formation. The EC50 value of Cl-IB-MECA induced inhibition of forskolin-stimulated cAMP accumulation was 11.7 ± 2.3 nM. In the presence of 1 and 3 μM VUF5455, the EC50 values were 7.4 ± 1.2 and 6.3 ± 0.8 nM, respectively, which were significantly different from that in the absence of VUF5455 (p < 0.05). It should be noted that VUF5455 might exert both allosteric enhancement and antagonistic effects in a functional assay. To avoid the confusing influence by the competitive inhibition, the effect of VUF5455 on the concentration-response curve for Cl-IB-MECA was investigated in the presence of the competitive antagonist MRS1220. Under this experimental condition, 10 μM VUF5455 caused a 3.2-fold shift to the left in the concentration-response relationship for Cl-IB-MECA (Fig. 9). The EC50 values obtained were 232 ± 67.4 and 72.5 ± 18.4 nM (n = 3) in the absence and presence of 10 μM VUF5455, respectively, which were significantly different (p < 0.05).

Fig. 6. Time course of the dissociation from [3H]-PIA dissociation from rat A1 adenosine receptors. Binding of 1 nM [3H]-PIA to A1 adenosine receptors in rat forebrain membranes was carried out at 37°C for 90 min in 50 mM Tris-HCl buffer, pH 7.7, in a total assay volume of 400 μl. The dissociation was begun by addition of 10 μM CPA. Samples were filtered after incubation at 37°C for 90 min in 400 μl of 50 mM Tris-HCl, pH 7.7, containing 10 mM MgCl2. NECA (10 μM) was used to define nonspecific binding. Dissociation was initiated by addition of 10 μM NECA before the preassociation. After 1-h preassociation, dissociation was initiated by addition of NECA (final concentration, 30 μM) and tested agents. The incubation was terminated by filtration through GF/B filters on a Brandel cell harvester. The data shown were derived from one experiment performed in duplicate and are typical of three independent experiments giving similar results.

Fig. 7. Time course of dissociation of [3H]CGS21680 from rat A1 adenosine receptors. Rat striatal membranes (80 μg) were incubated with 15 nM [3H]CGS21680 at 25°C for 90 min in 400 μl of 50 mM Tris-HCl, pH 7.7, containing 10 mM MgCl2. NECA (10 μM) was used to define nonspecific binding. Dissociation was started by the addition of 10 μM NECA in the presence and absence of tested compounds. The data shown were from one experiment performed in duplicate and are representative of three independent experiments giving similar results.

Fig. 8. Time course of the dissociation of [125I]I-AB-MECA dissociation from rat A3 adenosine receptors. [125I]I-AB-MECA (0.15 nM) was preassociated with the rat A3 adenosine receptor membranes (60 μg of protein) at 37°C for 1 h. Nonspecific binding was determined in parallel by addition of 30 μM NECA before the preassociation. After 1-h preassociation, dissociation was initiated by addition of NECA (final concentration, 30 μM) and tested agents. The incubation was terminated by filtration through GF/B filters on a Brandel cell harvester. The data shown were derived from one experiment performed in duplicate and are typical of three independent experiments giving similar results.
Discussion

This study provided evidence that several 3-(2-pyridinyl)isoquinoline derivatives were allosteric enhancers of the $\alpha_3$ adenosine receptor. The allosteric interaction was shown by the slowing of the dissociation of the agonist radioligand $[^{125}\text{I}]$-AB-MECA from $\alpha_3$ adenosine receptors.

The 3-(2-pyridinyl)isoquinoline derivatives were discovered as potential antagonists for $\alpha_3$ adenosine receptors (van Muijlwijk-Koezen et al., 1998). Previous studies demonstrated that the 3-(2-pyridinyl)isoquinoline derivative VUF58505 was a moderately potent and selective competitive antagonist for $\alpha_3$ adenosine receptors (van Muijlwijk-Koezen et al., 1998). The present study further demonstrated that this compound acts on human $\alpha_3$ adenosine receptors via the competitive binding site, but not an allosteric site.

VUF5804 was approximately 2 orders of magnitude more potent than VUF5455 in displacing $[^{125}\text{I}]$-AB-MECA binding, whereas the allosteric potencies of the two compounds were similar. A benzamide carbonyl group on the isoquinoline derivatives (Fig. 1A) was required for allosteric enhancement of $\alpha_3$ agonist binding, whereas the corresponding imines (Fig. 1B) did not enhance $\alpha_3$ agonist binding. These results showed that there were distinct structural requirements for allosteric enhancement of $\alpha_3$ adenosine receptor binding, and these requirements were different from those for competitive $\alpha_3$ antagonistic activity. For these two derivatives, species differences in $\alpha_3$ antagonist potency were pronounced, whereas the allosteric effects were comparable in rat and human. Apparently, it was possible to achieve some separation between allosteric and antagonistic activity of these compounds.

The compounds described in this study may serve as the first generation of allosteric enhancers for $\alpha_3$ adenosine receptors. Allosteric enhancers of $\alpha_3$ adenosine receptors may be of potential clinical use; however, application of the modulators as described in this study may have several limitations. An obvious factor that may limit the use of these agents is their antagonistic activities, which will tend to suppress any enhancing actions of these compounds. However, it seems that the structure-activity relationships for allosteric enhancement are separable from those for competitive antagonism, suggesting that it may be possible to discover compounds with improved enhancing activity that lack antagonist activity. Other limitations of these compounds include their low aqueous solubility (around 50 $\mu$M) and their modest allosteric potencies. Furthermore, the specificity of these compounds for adenosine receptors compared with other receptors has not been investigated. Hence, the possibility of other interfering activities cannot be excluded.

The identification of allosteric sites on receptors offers new pharmacological approaches to modulate receptor function. Although it is not known whether an allosteric site is a general feature of the GPCR family, increasing numbers of GPCRs are reported to be modulated allosterically by certain compounds. These include: metabotropic glutamate (Litschig et al., 1999), $\text{Ca}^{2+}$ (Conigrave et al., 2000), P2Y (Nepveu et al., 1998; Conigrave et al., 2000), muscarinic (Holzgrabe and Mohr, 1998), adenosine (Kourounakis et al., 2000), adrenergic (Leppik et al., 2000), dopamine (Hoare et al., 2000), 5-hydroxytryptamine (Thomas et al., 1997), tachykinin (Knaus et al., 1991; Croci et al., 1998), angiotensin AT$_1$ (Purdy et al., 1993), and oxytocin receptors (Grazzini et al., 1998). Although no GPCR allosteric enhancer is presently in clinical use, much progress has been made toward the allosteric modulation of this receptor family. Muscarinic receptor researchers currently use the radiolabeled allosteric ligand W84 as a probe to characterize the allosteric site on that receptor (Trankanl et al., 1998). Allosteric enhancers were found with almost absolute selectivity for certain subtypes of muscarinic receptors (Birdsall et al., 2001). This subtype selectivity was considered very difficult to achieve otherwise by targeting the recognition site on the receptor (Tucek and Proska, 1995). The allosteric enhancers of adenosine receptors have a potential advantage as therapeutic agents over adenosine receptor agonists [i.e., they seem less likely to cause desensitization and down-regulation of the receptor (Bhatatharya and Linden, 1996)]. The concept that an allosteric recognition site might serve as an alternate means to effect receptor activation was proposed and demonstrated for several G protein-coupled receptors, including muscarinic (Jakubik et al., 1996), 5-hydroxytryptamine$_2$ (Thomas et al., 1997), P2Y (Conigrave et al., 2000), and $\text{Ca}^{2+}$ receptors (Conigrave et al., 2000; Kobilka, 2000).

In the absence of mutation data for the allosteric effect, it is not yet possible to locate the allosteric site or even to speculate about the distance between and uniqueness of allosteric and orthosteric ligand binding sites on the $\alpha_3$ receptor. Nevertheless, there may be some structural interdependency between the two sites, which is indicated also by the fact that the same chemical class of ligands may bind at both.

For the $\alpha_1$ receptor, a single mutation of T277A, had effects on both (i.e., the affinity of agonists greatly diminished and orthosteric recognition site might serve as an alternate means to effect receptor activation was proposed and demonstrated for several G protein-coupled receptors, including muscarinic (Jakubik et al., 1996), 5-hydroxytryptamine$_2$ (Thomas et al., 1997), P2Y (Conigrave et al., 2000), and $\text{Ca}^{2+}$ receptors (Conigrave et al., 2000; Kobilka, 2000).

In the absence of mutation data for the allosteric effect, it is not yet possible to locate the allosteric site or even to speculate about the distance between and uniqueness of allosteric and orthosteric ligand binding sites on the $\alpha_3$ receptor. Nevertheless, there may be some structural interdependency between the two sites, which is indicated also by the fact that the same chemical class of ligands may bind at both.

For the $\alpha_1$ receptor, a single mutation of T277A, had effects on both (i.e., the affinity of agonists greatly diminished and orthosteric recognition site might serve as an alternate means to effect receptor activation was proposed and demonstrated for several G protein-coupled receptors, including muscarinic (Jakubik et al., 1996), 5-hydroxytryptamine$_2$ (Thomas et al., 1997), P2Y (Conigrave et al., 2000), and $\text{Ca}^{2+}$ receptors (Conigrave et al., 2000; Kobilka, 2000).

In the absence of mutation data for the allosteric effect, it is not yet possible to locate the allosteric site or even to speculate about the distance between and uniqueness of allosteric and orthosteric ligand binding sites on the $\alpha_3$ receptor. Nevertheless, there may be some structural interdependency between the two sites, which is indicated also by the fact that the same chemical class of ligands may bind at both.

For the $\alpha_1$ receptor, a single mutation of T277A, had effects on both (i.e., the affinity of agonists greatly diminished and orthosteric recognition site might serve as an alternate means to effect receptor activation was proposed and demonstrated for several G protein-coupled receptors, including muscarinic (Jakubik et al., 1996), 5-hydroxytryptamine$_2$ (Thomas et al., 1997), P2Y (Conigrave et al., 2000), and $\text{Ca}^{2+}$ receptors (Conigrave et al., 2000; Kobilka, 2000).

In the absence of mutation data for the allosteric effect, it is not yet possible to locate the allosteric site or even to speculate about the distance between and uniqueness of allosteric and orthosteric ligand binding sites on the $\alpha_3$ receptor. Nevertheless, there may be some structural interdependency between the two sites, which is indicated also by the fact that the same chemical class of ligands may bind at both.

For the $\alpha_1$ receptor, a single mutation of T277A, had effects on both (i.e., the affinity of agonists greatly diminished and orthosteric recognition site might serve as an alternate means to effect receptor activation was proposed and demonstrated for several G protein-coupled receptors, including muscarinic (Jakubik et al., 1996), 5-hydroxytryptamine$_2$ (Thomas et al., 1997), P2Y (Conigrave et al., 2000), and $\text{Ca}^{2+}$ receptors (Conigrave et al., 2000; Kobilka, 2000).
with other evidence, suggests that the allosteric site for some modulators may be close to the orthosteric site. The allosteric site of muscarinic receptors is thought to be near the exofacial surface of the receptor (Christopoulos et al., 1998).

In summary, allosteric modulators for A₃ adenosine receptors were identified and characterized. All of the compounds that showed allosteric enhancement also showed competitive binding properties. However, structure-activity relationships suggested the allosteric effects were separable from competitive antagonism. Hence, it may be possible to find allosteric enhancers that lack antagonistic properties by modifying the structures of these compounds or by screening compounds of other chemical entities.

Acknowledgments

We thank Miriam Dissen, Jacobien von Firstag Drabbe Künzel, and Neli Melman for their assistance with the ligand binding experiments.

References


Address correspondence to: Dr. K. A. Jacobson, Chief, Molecular Recognition Section, Bldg. 8A, RM B1A-19, NIH, NIDDK, LBC, Bethesda, MD 20892-0810. E-mail: kajaocbs@helix.nih.gov

Allosteric Modulation of A₃ Adenosine Receptors 1063
