Functional Characterization of Adenosine Receptors and Coupling to ATP-Sensitive K⁺ Channels in Guinea Pig Urinary Bladder Smooth Muscle

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ABSTRACT

Although multiple adenosine receptors have been identified, the subtype and underlying mechanisms involved in the relaxation response to adenosine in the urinary bladder remain unclear. The present study investigates changes in the membrane potential, as assessed by fluorescence-based techniques, of bladder smooth muscle cells by adenosine receptor agonists acting via ATP-sensitive potassium (K<sub>ATP</sub>) channels. Membrane hyperpolarization evoked by adenosine and various adenosine receptor subtype-selective agonists was attenuated or reversed by the K<sub>ATP</sub> channel blocker gliburide. Comparison of adenosine receptor agonist potencies eliciting membrane potential effects showed a rank order of potency 5'-N-ethylcarboxamido adenosine (NECA; log EC<sub>50</sub> = 7.97) ~ 2-p-(2-carboxethyl)phenethyl-amino-5'-N-ethylcarboxamidoadenosine hydrochloride (CGS-21680; 7.65) > 2-chloro adenosine (5.90) ~ 2-chloro-N<sup>6</sup>-cyclopentyladenosine (CCPA; 5.51) ~ N<sup>6</sup>-cyclopentyladenosine ~ N<sup>6</sup>-(R)-phenylisopropyladenosine > 2-chloro-N<sup>6</sup>-(3-iodobenzyl)-adenosine-5'-N-methyl-carboxamido (2CI-IB-MECA; 4.78). Membrane potential responses were mimicked by forskolin, a known activator of adenylyl cyclase, and papaverine, a phosphodiesterase inhibitor. The A<sub>2A</sub>-selective antagonist 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-yl-amino]ethyl)phenol (ZM-241385), and the adenylyl cyclase inhibitor N-(cis-2-phenyl-cyclopropyl) azacitroclidecan-2-imine-hydrochloride (MDL-12330A) inhibited the observed change in membrane potential evoked by adenosine and adenosine-receptor agonists. The rank order potency for relaxation of K⁺-stimulated guinea pig bladder strips, NECA (log EC<sub>50</sub> = 6.41) ~ CGS-21680 (6.38) > 2-chloro adenosine (5.90) > CCPA ~ 2CI-IB-MECA (>4.0) was comparable to that obtained from membrane potential measurements. Collectively, these studies demonstrate that adenosine-evoked membrane hyperpolarization and relaxation of bladder smooth muscle is mediated by A<sub>2A</sub> receptor-mediated activation of K<sub>ATP</sub> channels via adenylyl cyclase and elevation of cAMP.

Adenosine is an endogenous nucleoside that regulates many physiological functions in cardiovascular, respiratory, renal, immune, and central and peripheral nervous systems via G protein-coupled adenosine receptors (P1 receptors). Four receptor subtypes, A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub>, and A<sub>3</sub>, have been identified based on molecular structure, pharmacology, and mechanisms of G-protein-mediated signaling mechanisms (for reviews, see Ralevic and Burnstock, 1998; Fredholm et al., 2000). The A<sub>1</sub> receptors are coupled to the inhibition of adenylyl cyclase and can act through effector pathways such as stimulation of phospholipase C, activation of potassium channels, and inhibition of N-type calcium channels. The A<sub>2</sub> receptor subtypes, A<sub>2A</sub> and A<sub>2B</sub>, are coupled to activation of adenylyl cyclase, whereas A<sub>3</sub> receptors has been shown to stimulate phospholipase C and D and to inhibit adenylyl cyclase (Abbracchio et al., 1995; Baraldi et al., 2000).

In the past decade, considerable effort has been invested in elucidating the physiological roles of the various adenosine receptors in a variety of tissues, including cardiac, vascular, and nonvascular tissues. Adenosine and adenosine receptors are thought to play a critical role in regulating urinary bladder function, especially in conditions of bladder ischemia or during obstruction-induced changes in function that underlie

ABBREVIATIONS: K<sub>ATP</sub> channel, ATP-sensitive K⁺ channel; FLIPR, fluorescent imaging plate reader; ZM-241385; 4-[2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-yl-amino]ethyl]phenol; MECA, 5'-N-methylcarboxamido adenosine; P1075, N-cyano-N'-(1,1-dimethylpropyl)-N'-3-pyridylguanidine; CPA, N<sup>6</sup>-cyclopentyladenosine; CCPA, 2-chloro-N<sup>6</sup>-cyclopentyladenosine; R-PIA, (R)-N<sup>6</sup>-phenylisopropyladenosine; CGS-21680, 2-p-(2-carboxethyl)phenethyl-amino-5'-N-ethylcarboxamidoadenosine hydrochloride; NECA, 5'-N-ethylcarboxamido adenosine; CPCPA, 5'-N-cyclopentylcarboxamido adenosine; DPCPX, 8-cyclopentyl-3,7-dihydro-1,3-dipropyl-1H-purine-2,6-dione; CGS-15943, 9-chloro-2-(2-furyl)[1,2,4]triazolo[1,5-c]quinazolin-5-amine; MDL-12330A, N-(cis-2-phenyl-cyclopropyl) azacitroclidecan-2-imine-hydrochloride; 2CI-IB-MECA, 2-chloro-N<sup>6</sup>-(3-iodobenzyl)-adenosine-5'-N-methyl-carboxamido.
lower urinary tract symptoms (Levin et al., 2000). Although evidence for inhibitory P1-type purinergic receptor-mediated effects by adenosine has been reported in the bladder (Nicholls et al., 1992; King et al., 1997), the receptor subtype(s) and their coupling to smooth muscle relaxation events remain poorly understood. mRNA analysis by Dixon et al. (1996) has revealed the presence of subunits corresponding to all adenosine receptor subtypes in the rat bladder, whereas Northern analysis showed relatively higher expression of the A_2B receptor subtype (Stehle et al., 1992). Based on the order of potency of adenosine agonists for inhibition of carbachol-evoked contractions in rat bladder, it was noted that the pharmacological profile was more consistent with the participation of adenosine A_2 receptor subtypes (Nicholls et al., 1992, 1996). On the other hand, pharmacological analysis of smooth muscle cells isolated from the circular muscle layer of the feline bladder showed that the adenosine receptor subtype mediating contraction resembled that of the A_1 subtype in this species (Yang et al., 2000). In vascular smooth muscle, adenosine interacts with both A_1 and A_2 receptor subtypes to activate K_ATP channels leading to relaxation. In the rabbit mesenteric artery, A_2 receptor subtypes are predominantly involved, whereas in pig coronary arteries, adenosine-evoked activation occurs via A_1 receptors (Quayle et al., 1997). However, it remains unclear which adenosine receptor subtype mediates functional responses in the bladder and whether functional coupling of these receptors to K_ATP channels might provide a mechanism underlying bladder smooth muscle relaxation.

In the present study, we have investigated the nature of the adenosine receptor subtype(s) involved in coupling to K_ATP channels in the guinea pig urinary bladder, a widely used model for studying the physiology of bladder function (Fuji et al., 1990; Herrera et al., 2000). Our studies provide evidence that hyperpolarization of membrane potential via K_ATP channel activation is a necessary component of adenosine receptor activation. Based on pharmacological analysis of membrane potential changes and tissue relaxation effects, it was found that adenosine-mediated relaxation of the guinea pig bladder involves the A_2A receptor subtype, which appears to be linked to K_ATP channel activation by activation of adenylate cyclase.

**Experimental Procedures**

**Materials.** Cell culture reagents were purchased from Invitrogen (Carlsbad, CA). The fluorescent imaging plate reader (FLIPR) membrane potential kit was purchased from Molecular Devices (product number R-8034; Sunnyvale, CA). Glyburide (glibenclamide), adenosine, adenosine deaminase, and other adenosine agonists and antagonists, forskolin, and papaverine were purchased from Sigma Chemical (St. Louis, MO). ZM-241385 and 2Cl-IBMECA were purchased from Tocris Cookson Inc. (Ballywin, MO). N-Cyano-N’-(1,1-dimethylpropyl)-N’-3-pyridylguanidine (P1075) was synthesized in house. Compounds were prepared as 10 mM stocks in dimethyl sulfoxide and diluted in assay buffer just before use.

**Cell Culture.** Urinary bladder smooth muscle cells were prepared as previously described (Gopalakrishnan et al., 1999; Whiteaker et al., 2001). Bladders were stripped of serosa and mesentery. Bladders were collected from animals weighing 250 to 300 g and immediately placed in Krebs-Ringer bicarbonate solution (composition 120 mM NaCl, 20 mM NaHCO_3, 11 mM dextrose, 4.7 mM KCl, 1.2 mM KH_2PO_4, 0.01 mM K_2EDTA, equilibrated with 5% CO_2, 95% O_2; pH 7.4 at 37°C). The trigonal and dome portions were discarded and strips 3 to 5 mm in width and 10 mm in length were prepared from the remaining tissue by cutting in a circular manner. One end of the strip was fixed to a stationary glass rod and the other to a Grass FT03 transducer at a basal preload of 1.0 g. This preload proved to be the best condition for a steady-state baseline and reproducible responses to K⁺ stimulation. As previously reported (Herrera et al., 2000; Buckner et al., 2002), the contractions evoked by mild depolarization (i.e., 20 mM K⁺) are largely myogenic in origin as revealed by the lack of sensitivity to the sodium channel blocker tetrodotoxin (0.1 μM). Tissues were allowed to equilibrate for at least 60 min before the assay. Concentration-response curves were generated in a noncumulative manner with a rise between successive additions.

**Data Analysis.** In FLIPR studies, the fluorescence responses were corrected for any background changes in the negative control wells, and data were normalized to those observed with 10 mM adenosine (100% assigned to maximum decrease in fluorescence units). In tissue bath assays, responses were measured as decreases in tension and expressed as percentage of tension evoked by 20 mM K⁺. Data are expressed as mean ± S.E.M. The EC₅₀ values were calculated from the concentration-response curve generated by nonlinear regression analysis (GraphPad Prism; GraphPad Software, San Diego, CA). Significant differences between group means were assessed by analysis of variance followed by Student-Newman-Keuls test. Significance was accepted at the P < 0.05 level.

**Results**

**Effect on Membrane Potential Responses in Bladder Smooth Muscle Cells**

Adenosine evoked concentration-dependent decreases in fluorescence responses in guinea pig urinary bladder smooth...
Effects of adenosine in guinea pig bladder smooth muscle cells. Data are normalized to the maximal responses evoked by adenosine (10 μM, inset). Significant changes in membrane potential responses were noted at concentrations as low as 10 nM and maximal responses were obtained with 10 μM adenosine. The EC₅₀ value was calculated to be 467 nM (±log EC₅₀ = 6.33 ± 0.11; n = 9). Adenosine responses were reversed by the addition of 10 μM glyburide or attenuated by glyburide pretreatment (see below), suggesting that these effects could be mediated by activation of Kₐtp channels. Glyburide alone did not alter the basal fluorescence response (data not shown). The maximal efficacy of adenosine (94.9 ± 2.5%) was comparable to that of a prototypical Kₐtp channel opener such as the cyanoguanidine, P1075 under similar conditions.

Effect of Glyburide and Adenosine Deaminase Treatments

To further investigate the involvement of Kₐtp channels, smooth muscle cells were pretreated with glyburide for 30 min before addition of adenosine. As shown in Fig. 2, glyburide pretreatment significantly attenuated the response of adenosine in a concentration-dependent manner. To examine whether the effects of adenosine are directly mediated, experiments were performed in the presence of adenosine deaminase, which catalyzes deamination of adenosine to inosine. Addition of varying concentrations of adenosine (0.03–10 μM) along with adenosine deaminase (0.03 units/ml) completely abolished the responses of adenosine, which demonstrates that adenosine activates Kₐtp channels by itself, and not via its catabolic products.

Effect of Adenosine Receptor Agonists

To address the nature of the adenosine receptor(s) involved in this phenomenon, the effects of various adenosine receptor agonists (Jacobsen et al., 1992; Jacobson and Knutsen, 2001) were evaluated. In each case, agonist-evoked reductions in fluorescence potential effects were attenuated by glyburide.

Adenosine A₁ Agonists. The N⁶-substituted adenosine derivatives, including N⁶-cyclopentyladenosine (CPA, A₁ Kᵢ = 2.3 nM) and its analog 2-chloro-N⁶-cyclopentyladenosine (CCPA) and N⁶-(R)-phenylisopropyladenosine (R-PIA; A₁ Kᵢ = 2 nM) are selective agonists at A₁ receptors (Jacobson and Knutsen, 2001). The kinetic data of CCPA are shown in Fig. 3A. The concentration-response curves for changes in fluorescence responses by CCPA and other A₁ receptor agonists are provided in Fig. 3, B and C. Although the efficacies of these compounds were comparable to that of adenosine, the EC₅₀ values were generally in the micromolar range (Table 1). 2-Chloro-adenosine, a stable analog of adenosine that is only about 7-fold more selective for the A₁ receptor (Kᵢ = 9 nM) compared with A₂aA₂b receptor subtypes (Fredholm et al., 1994), also elicited concentration-dependent changes in membrane potential responses.

Adenosine A₂aA₂b Agonists. Next, the effects of CGS-21680, a compound that is approximately 170-fold selective for the A₂a versus A₁ and (A₂a Kᵢ = 15 nM; A₁ Kᵢ = 2800 nM) with weak affinity at A₂b receptors was examined together with effects of the nonselective agonist NECA (Hutchinson et al., 1990; Jacobson and Knutsen, 2001). Both CGS-21680 and

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**Fig. 1.** Concentration-dependent changes in fluorescence responses evoked by adenosine in guinea pig bladder smooth muscle cells. Representative traces showing decreases in responses (fluorescence units) to varying concentrations of adenosine (0.003, 0.03, 0.3, 3, 10, and 30 μM) and its reversal upon addition of the Kₐtp channel blocker glyburide. Data are provided in Table 1, which compares the efficacies of various adenosine receptor agonists.

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**Fig. 2.** Inhibition of adenosine-mediated responses in bladder smooth muscle cells by glyburide and adenosine deaminase. Shown are responses to adenosine in the absence and presence of glyburide (3 and 30 μM) or adenosine deaminase. Cells are pretreated with glyburide for 30 min and then challenged with adenosine. In studies with adenosine deaminase, adenosine at various concentrations was added in the presence of adenosine deaminase (final concentration of 0.03 units/ml). Shown are means ± S.E.M. of at least three separate determinations.

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**Fig. 3.** Effects of adenosine A₁ receptor agonists in bladder smooth muscle cells. A, typical concentration-dependent reductions in fluorescence responses evoked by A₁-selective agonist CCPA and its reversal after the addition of glyburide. Concentration-response profile of adenosine receptor agonists CPA, R-PIA, and CCPA (B) and phenyl ethyl adenosine, 2-chloroadenosine, and adenosine amine congener (ADAC, C). Shown are means ± S.E.M. of at least three separate determinations.
TABLE 1
Membrane potential responses evoked by adenosine receptor agonists in guinea pig bladder smooth muscle cells

<table>
<thead>
<tr>
<th>Compound</th>
<th>EC_{50} (M)</th>
<th>-log EC_{50}</th>
<th>Efficacy</th>
<th>Slope</th>
<th>n</th>
</tr>
</thead>
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<tr>
<td>Adenosine</td>
<td>0.47 ± 0.07</td>
<td>6.33 ± 0.11</td>
<td>94.9 ± 2.5</td>
<td>1.20 ± 0.16</td>
<td>9</td>
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<tr>
<td>2-Chloroadenosine</td>
<td>1.09 ± 0.19</td>
<td>5.96 ± 0.07</td>
<td>75.7 ± 7.7</td>
<td>1.42 ± 0.09</td>
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<tr>
<td>NECA</td>
<td>0.011 ± 0.008</td>
<td>7.97 ± 0.23</td>
<td>102.8 ± 8.0</td>
<td>1.41 ± 0.46</td>
<td>9</td>
</tr>
<tr>
<td>A_1 Receptor</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CPA</td>
<td>2.00 ± 0.57</td>
<td>5.70 ± 0.11</td>
<td>82.9 ± 10.2</td>
<td>2.08 ± 0.59</td>
<td>3</td>
</tr>
<tr>
<td>CCPA</td>
<td>3.09 ± 1.41</td>
<td>5.51 ± 0.19</td>
<td>84.4 ± 1.3</td>
<td>2.58 ± 0.95</td>
<td>3</td>
</tr>
<tr>
<td>R-PIA</td>
<td>3.16 ± 1.48</td>
<td>5.50 ± 0.20</td>
<td>88.7 ± 15.4</td>
<td>2.00 ± 1.46</td>
<td>3</td>
</tr>
<tr>
<td>ADAC</td>
<td>2.95 ± 0.72</td>
<td>5.53 ± 0.12</td>
<td>80.4 ± 20.0</td>
<td>1.43 ± 0.12</td>
<td>3</td>
</tr>
<tr>
<td>N^6-2-Phenyl ethyl adenosine</td>
<td>1.02 ± 0.19</td>
<td>5.99 ± 0.08</td>
<td>76.9 ± 3.3</td>
<td>1.48 ± 0.09</td>
<td>3</td>
</tr>
<tr>
<td>A_{2A}/A_{1B} Receptor</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CGS-21680</td>
<td>0.022 ± 0.007</td>
<td>7.65 ± 0.17</td>
<td>98.8 ± 6.7</td>
<td>1.68 ± 0.21</td>
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</tr>
<tr>
<td>2-Phenylamino adenosine</td>
<td>0.185 ± 0.013</td>
<td>6.71 ± 0.03</td>
<td>106.6 ± 6.7</td>
<td>1.10 ± 0.31</td>
<td>3</td>
</tr>
<tr>
<td>MECA</td>
<td>0.29 ± 0.10</td>
<td>6.53 ± 0.15</td>
<td>95.9 ± 13.5</td>
<td>1.28 ± 0.14</td>
<td>3</td>
</tr>
<tr>
<td>CPCIA</td>
<td>0.018 ± 0.001</td>
<td>7.73 ± 0.03</td>
<td>102.8 ± 5.6</td>
<td>2.31 ± 0.30</td>
<td>3</td>
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<tr>
<td>A_3 Receptor</td>
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<td></td>
</tr>
<tr>
<td>2CI-IBMECA</td>
<td>16.60 ± 9.3</td>
<td>4.78 ± 0.23</td>
<td>105.9 ± 6.3</td>
<td>0.87 ± 0.07</td>
<td>3</td>
</tr>
<tr>
<td>AB-MECA</td>
<td>8.13 ± 2.34</td>
<td>5.09 ± 0.14</td>
<td>94.9 ± 6.2</td>
<td>2.90 ± 0.62</td>
<td>3</td>
</tr>
<tr>
<td>N^6-Benzyl NECA</td>
<td>0.46 ± 0.11</td>
<td>6.35 ± 0.10</td>
<td>102.2 ± 2.3</td>
<td>1.10 ± 0.11</td>
<td>3</td>
</tr>
</tbody>
</table>

Fig. 4. Effects of adenosine A_1 receptor agonists in bladder smooth muscle cells. Shown are concentration-dependent reductions in glyburide-sensitive fluorescence responses evoked by A_2A-selective agonist CGS-21680 (A) and A_3 agonist NECA (B). C, concentration-response profile of CGS-21680, NECA, CPCIA, MECA, and phenylaminoadenosine. Shown are means ± S.E.M. of at least three separate determinations.

MECA (K_i = 1.39 nM; Jacobson et al., 1993) and N^6-benzyl NECA (K_i = 6.8 nM; van Galen et al., 1994) were effective, but considerably less potent compared with their affinities reported at the A_3 receptor subtype (Fig. 5B; Table 1).

Effect of Adenosine Receptor Antagonists

To further investigate the adenosine receptor subtype involved in K_{ATP} channel activation, the effects of selective receptor antagonists were examined.

Inhibition of Adenosine-Mediated Responses by Selective Antagonists. DPCPX, an adenosine antagonist with about 500-fold greater selectivity for the rodent A_1 (K_i = 0.46 nM) than A_2 receptors (Halleen et al., 1987), was used to assess the involvement of the A_1 receptor. As shown in Fig. 6A, 100 nM DPCPX did not alter adenosine-evoked changes in membrane potential. CGS-15943, another compound that is about 100-fold more selective in antagonizing A_2A receptor compared with A_2B, but only about 10-fold selective versus the A_2A subtype (Ongini et al., 1999) also inhibited responses of adenosine (0.3 and 1 M). The effects of ZM-241385, an A_2A receptor-selective compound with 30- to 80-fold greater selectivity for the A_2A receptor subtype compared with the A_2B receptor and 1000-fold selectivity compared with the A_1 receptor (Poucher et al., 1995), was also examined. As shown
volvement of A2A receptors in activating K<sub>ATP</sub> channels in the bladder smooth muscle.

**Coupling of Adenosine A2A Receptors to K<sub>ATP</sub> Channels**

The observation that adenosine activates glyburide-sensitive changes in membrane potential by activation of A2A receptors suggests a role for the involvement of intracellular cAMP and subsequent activation of protein kinase A. To assess the nature of coupling of A2A receptors to K<sub>ATP</sub> channels, agents that modulate adenylate cyclase and/or cAMP levels were examined. First, the effect of A2A receptors to K<sub>ATP</sub> channels, agents that modulate adenylate cyclase and/or cAMP levels were examined. First, the effect of A2A receptors to K<sub>ATP</sub> channels, agents that modulate adenylate cyclase and/or cAMP levels were examined. First, the effect of K<sub>ATP</sub> channels in bladder smooth muscle cells by elevation of intracellular cAMP and stimulation of protein kinase A. Consistent with these observations, direct activation of cAMP levels by forskolin also resulted in concentration-dependent decreases in fluorescence responses that were reversed by glyburide (Fig. 8B). In addition to forskolin, papaverine, a phosphodiesterase inhibitor that elevates cellular cAMP levels by preventing its breakdown, was also found to be effective in activating K<sub>ATP</sub> channels.

**Adenosine Agonist-Evoked Relaxation of Bladder Strips**

The inhibitory effects of adenosine receptor ligands were evaluated in bladder smooth muscle strips precontracted with 20 mM K<sup>+</sup>. It is known that spontaneous phasic contractility of the bladder smooth muscle are largely myogenic in nature, insensitive to suppression by 100 nM tetrodotoxin, but inhibited by L-type calcium channel blockers and K<sub>ATP</sub> channel openers (Fujii et al., 1990; Herrera et al., 2000; Buckner et al., 2002). As shown in Fig. 9, the nonselective agonist NECA and other A2A receptor-selective agonists, CGS-21680, CPCA, and 2-phenylaminoadenosine, inhibited bladder contractions with -log IC<sub>50</sub> values of 6.41 ± 0.05, 6.38 ± 0.22, 6.91 ± 0.15, and 5.53 ± 0.21, respectively (Table 2). In contrast, the A1-selective agonist CCPA and the A3-selective agonist 2Cl-IBMECA did not inhibit contractions, even at the highest concentrations (100 μM) tested. 2-Chloroadenosine also suppressed phasic contractility (-log IC<sub>50</sub> = 5.92 ± 0.32), which could be attributed to an effect at the A2 receptor subtypes. The rank order potencies of adenosine

![Fig. 6](image-url). Effect of adenosine receptor antagonists. Responses of varying concentrations of adenosine (0.3, 1, and 3 μM) in the absence and presence of the A<sub>1</sub> receptor antagonist DPCPX (100 nM) (A) or in presence of an A<sub>2A</sub> receptor antagonist CGS-15943 (50 nM) (B). Shown are means ± S.E.M. of at least three separate determinations. *, values significantly different from responses evoked by adenosine alone.

![Fig. 7](image-url). Effect of A<sub>2A</sub> receptor antagonists on adenosine and NECA-mediated effects. A, responses of 3 μM adenosine (denoted by C) or with varying concentrations of ZM-241385 (0.1, 1, and 10 μM indicated along the x-axis) or alloxazine (1 and 10 μM). B, responses of 1 μM NECA alone (denoted by C) or with varying concentrations of ZM-241385 (0.1, 1, and 10 μM) indicated along the x-axis) or alloxazine (1 and 10 μM). Shown are means ± S.E.M. of at least three separate determinations. *, values significantly different from responses evoked by adenosine alone.

![Fig. 8](image-url). Effects of adenylate cyclase modulation on adenosine-evoked membrane potential responses. A, concentration-dependent changes in adenosine responses in the absence and presence of MDL-12330A (10 and 100 μM). Cells were treated with the adenylate cyclase inhibitor MDL-12330A before addition of adenosine. B, concentration-dependent changes in fluorescence responses evoked by forskolin and papaverine. Data are normalized to the decrease in fluorescence responses evoked by 10 μM adenosine.
agonists in suppressing phasic contractility of the bladder strips and for evoking membrane potential effects were comparable.

**Discussion**

The present study provides evidence that adenosine evokes membrane hyperpolarization in guinea pig bladder smooth muscle cells by activation of $K_{ATP}$ channels. Pharmacological characterization demonstrates that a variety of subtype-selective agonists of adenosine A2A receptor (NECA, CGS-21680, CPA, phenylaminoadenosine (CPA), A2-selective agonist (2CI-IBMECA), and 2-chloro-adenosine, a stable analog of adenosine (B)).

**Adenosine Receptor-Mediated Activation of $K_{ATP}$ Channels.** By using fluorescence-based readout of membrane potential changes, the present study shows that adenosine can hyperpolarize guinea pig bladder smooth muscle cells in a concentration-dependent manner. Fluorescence-based membrane potential assays of $K_{ATP}$ channel function serves as a sensitive method to evaluate changes in membrane potential with a response time comparable to ligand-gated channel activation measured by patch-clamp studies. Previous studies have shown that $K_{ATP}$ channels in bladder myocytes can be activated by P1075 and other structurally divergent $K_{ATP}$ channel openers with a rank order of potency that correlates well with relaxation of bladder smooth muscle strips (Gopalakrishnan et al., 1999; Whiteaker et al., 2001). The reversal of membrane potential effects by adenosine and subtype-selective agonists by glyburide demonstrates their activation of $K_{ATP}$ channels in the bladder smooth muscle.

**Pharmacological Characterization of Adenosine Receptors.** Functional analysis with subtype-selective agonists of $K_{ATP}$ channel-mediated membrane potential changes and relaxation responses support the participation of adenosine A2 receptor subtypes (Table 1). In particular, CGS-21680 that is selective for the A2A receptor subtype (Hutchinson et al., 1989; Jacobson and Knutzen, 2001) was found to be as potent as NECA in evoking membrane potential effects (Table 1). The rank order of potencies, NECA ($-\log EC_{50} = 7.97$) > CGS-21680 (7.65) > 2-chloro adenosine (5.90) > CPA ~ CPA ~ R-PIA > 2CI-IBMECA (4.78) is consistent with that defining the A2A receptor subtype (Feoktistov and Biaggioni, 1997). As shown in Figs. 3 and 5, agonists with reportedly nanomolar affinity at the A1 and A3 receptor subtype were found to affect membrane potential responses only at relatively higher concentrations (EC_{50} values in the micromolar range). Similarly, in tissue relaxation studies, NECA and CGS-21680 were equipotent in suppressing myogenic contractions of the bladder strips, whereas the A1 receptor-selective agonist (CPA) and A3-selective agonist (2CI-IBMECA) were inactive. Taken together, the data indicate that the A2A receptor subtype mediates adenosine-evoked relaxation of urinary bladder smooth muscle.

Further support for participation of the A2A receptor was derived from studies with selective antagonists. The concentration response of membrane potential effects evoked by adenosine was not shifted by the presence of DPCPX, suggesting the lack of participation of adenosine A1 receptors. On the other hand, ZM-241385, a selective A2A receptor antagonist, suppressed adenosine- and NECA-evoked hyperpolarization responses. In the absence of selective agonists or antagonists, A2B receptors have been typically characterized by the method of exclusion or lack of agonists that are specific to other subtypes. The lack of effect of low concentrations of alloxazine, together with the relative potency of selective agonists, suggests that A2B receptor may not likely be involved. Detailed analysis of mRNA expression by reverse transcription-polymerase chain reaction in rat urinary bladder have shown the presence of mRNA corresponding to A1, A2A, A2B, and A3 subtypes (Dixon et al., 1996). Although comparable studies in guinea pig bladder are currently unavailable, the rank order potency of agonists together with the suppression of adenosine or NECA-evoked membrane potential by ZM-241385 indicates that A2A receptor could be functionally involved in adenosine receptor signaling.

**Mechanism of Adenosine A2A Receptor Signaling.** The inhibition of adenosine effects by the adenylyl cyclase inhibitor MDL-123390A together with the observation that the effects can be mimicked by direct activation of adenylyl cyclase by forskolin or after inhibition of cAMP-dependent phosphodiesterase, in a glyburide-reversible manner, implicates that the coupling to $K_{ATP}$ channels involves elevation of cAMP. Although both A2A and A2B receptors are coupled through G_s protein to the activation of adenylyl cyclase and elevation of intracellular cAMP (Fredholm et al., 1994), the present study provide evidence that it is the A2A
receptor that is linked to the activation of K\textsubscript{ATP} channels in guinea pig bladder smooth muscle. This situation is comparable to that reported in the vasculature, as for example, in arterial myocytes where adenosine acts via the A\textsubscript{2A} receptor subtype and protein kinase A to stimulate K\textsubscript{ATP} currents (Kleppisch and Nelson, 1995). Adenosine-induced vasodilatation in other vascular beds, including the microvasculature of rat diaphragm and afferent arterioles in rat kidney, have also been shown to involve the A\textsubscript{2A} receptor, which are coupled to adenylate cyclase activation and opening of the K\textsubscript{ATP} channel (Tang et al., 1999; Chen et al., 2000).

A\textsubscript{2A} Receptor-K\textsubscript{ATP} Channel Interactions. K\textsubscript{ATP} channels have been shown to be hetero-octomeric complexes composed of four inward rectifying K\textsuperscript{+} channels belonging to the Kir 6.0 subfamily that forms the K\textsuperscript{+}-selective pore and four regulatory proteins, the sulfonylurea receptors. The latter serves to catalyze nucleotide binding and hydrolysis and hosts binding sites for K\textsubscript{ATP} channel openers and sulfonylurea blockers (Bryan and Aguilar-Bryan, 1999; Seino, 1999). On the basis of reverse transcription-polymerase chain reaction and pharmacological studies, it has been suggested that K\textsubscript{ATP} channels in guinea pig bladder smooth muscle may be derived from combinations of sulfonylurea receptor 2B and Kir6.2. Although our studies show that adenosine A\textsubscript{2A} receptor elevates cAMP and possibly activates protein kinase A-mediated phosphorylation processes, the mechanism by which this leads to activation of K\textsubscript{ATP} channels remains to be elucidated by future coexpression studies of the A\textsubscript{2A} receptor with K\textsubscript{ATP} channel subunits.

A large body of evidence shows that adenosine receptors can activate K\textsubscript{ATP} channels in vasculature, especially those for the coronary and mesenteric beds (Dart and Standen, 1993; Quayle et al., 1997). More recently, it has been shown that adenosine receptor activation can enhance the potency of cromakalim via A\textsubscript{2A} receptors, but not that of pinacidil (Dave et al., 1999). Although no comparable studies have been carried out in bladder smooth muscle, it raises the possibility that the sensitivity of K\textsubscript{ATP} channel openers could be enhanced under metabolically compromised conditions, where adenosine release is higher, as for example, in bladder instability subsequent to partial outlet obstruction. It has been reported that hypertrophied bladder secondary to partial outlet obstruction is more resistant to hypoxic ischemia and reperfusion damage than normal tissue (Levin et al., 1997). Although adenosine receptor subtypes have been widely exploited as therapeutic targets (Williams and Jarvis, 2000), the difficulty in separating therapeutic effects from potential side effects has somewhat dampened the development of drugs acting at adenosine receptors. Enhanced understanding of the roles and mechanisms underlying adenosine receptor activation and signaling in various tissues could facilitate future exploitation of the potential of targeting adenosine receptor subtypes. In summary, these present studies demonstrate that adenosine-evoked membrane hyperpolarization and relaxation of urinary bladder smooth muscle are mediated by adenosine A\textsubscript{2A} receptor-mediated activation of K\textsubscript{ATP} channels via adenylate cyclase and elevation of cAMP.

References


Buckner SA, Milicie A, Daza A, Coghlan MJ, and Gopalakrishnan M (2002) Spon-
taneous phasic contractility of the pig urinary bladder smooth muscle: characteris-

tion and pharmacological studies, it has been suggested that K\textsubscript{ATP} channel opening drugs by adenosine A\textsubscript{1} and A\textsubscript{2} receptors in the pig coronary artery. Eur J Pharmacol 383(1–2):155–162.


Jacobson KA and Knutzen LJS (2001) P1 and P2 purine and pyrimidine receptor ligands, in Handbook of Experimental Pharmacology–Purinergic and Pyrimidinergic Signalling I: Molecular, Nervous, and Urogenitary System Function (Abbrac-
chio MP and Williams M eds), vol 151, pp 129–175, Springer Verlag, New York.


Nicholls J, Brownhill VR, and Hourani SMO (1996) Characterization of P1 purino-


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