Determination of Adenosine Effects and Adenosine Receptors in Murine Corpus Cavernosum

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ABSTRACT

This study tested the hypothesis that adenosine, in murine corpora cavernosa, produces direct relaxation of smooth muscle cells and inhibition of contractile responses mediated by sympathetic nerve stimulation. Penes were excised from anesthetized male C57BL/6 mice, dissected, and cavernosal strips were mounted to record isometric force. Adenosine, 2-chloroadenosine (stable analog of adenosine), and 2-phenylaminoadenosine (CV1808) (A2A/A2B agonist) produced concentration-dependent relaxations of phenylephrine-contracted tissues. Relaxation to 2-chloroadenosine was inhibited, in a concentration-dependent manner, by 2-(2-furanyl)-7-(2-phenylethyl)-7H-pyrano[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-5-amine (SCH58261; A2A antagonist; 10⁻⁶–10⁻⁴ M) and N-(4-acetylphenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-8-yl)phenoxy]acetamide (MRS1706; A2B antagonist; 10⁻⁵–10⁻⁴ M). The combination of both antagonists abrogated 2-chloroadenosine-induced relaxation. Electrical field stimulation (EFS; 1–32 Hz) of adrenergic nerves produced frequency-dependent contractions that were inhibited by compounds that increase adenosine levels, such as 5'-iodotubercidin (adenosine kinase inhibitor), erythro-9-(2-hydroxy-3-nonyl)adenine (adenosine deaminase inhibitor), and dipyridamole (inhibitor of adenosine transport). The adenosine A1 receptor agonist N⁶-cyclopentyladenosine (C8031) right-shifted contractile responses to EFS, with a significant inhibitory effect at 10⁻⁶ M. Blockade of adenosine A1 receptors with 8-cyclopentyl-1,3-dipropylxanthine (C101) (10⁻⁷ M) enhanced contractile responses to EFS and eliminated the inhibitory effects of 5'-iodotubercidin. Dipyridamole and 5'-iodotubercidin had no effect on adenosine-mediated relaxation. In summary, adenosine directly relaxes cavernosal smooth muscle cells, by the activation of A2A/A2B receptor subtypes. In addition, adenosine negatively modulates sympathetic neurotransmission, by A1 receptor subtype activation, in murine corpora cavernosa. Adenosine may subserve dual roles in modulating the physiological mechanisms of erection in mice.

The balance between contractile and relaxing factors controls smooth muscle tone of both the penile vasculature and corpora cavernosa, and, therefore, it determines the functional state of the penis: flaccidity or erection (Andersson, 2001; Leite et al., 2007). Efferent autonomic sympathetic and parasympathetic nerves regulate penile function, via release of norepinephrine and acetylcholine, respectively. In addition, many investigators have provided evidence for functional roles of nonadrenergic-noncholinergic (NANC) inhibitory and excitatory nerves, which contain not only neuropeptides, such as neuropeptide Y, vasoactive intestinal polypeptide, substance P, and calcitonin gene-related polypeptide, but also transmitters such as nitric oxide (NO) and adenosine triphosphate (ATP) (Giuliano and Rampin, 2004). ATP, which is also a cotransmitter in most adrenergic nerves (Lundberg, 1996; Burnstock, 2006), is broken down by extracellular ATPases and 5'-nucleotidase.

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ABBREVIATIONS: NANC, nonadrenergic-noncholinergic; l-NAME, N⁶-nitro-l-arginine methyl ester; EHNA, erythro-9(2-hydroxy-3-nonyl)adenine; C8031, N⁶-cyclopentyladenosine; C101, 8-cyclopentyl-1,3-dipropylxanthine; MRS1706, N-(4-acetylphenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-8-yl)phenoxy]acetamide; SCH58261, 2-(2-furanyl)-7-(2-phenylethyl)-7H-pyrano[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-5-amine; CV1808, 2-phenylaminoadenosine; 5'-iodotubercidin, 4-amino-5-iodo-7-(β-D-ribofuranosyl)pyrrolo[2,3-d]pyrimidine; dazep dihydropyridine; 1,4-bis(3,4,5-trimethoxybenzoyloxy)propyl]homopiperazine dihydrochloride; dipyridamole, 2,6-bis(diethanolamino)-4,8-dipiperidinoypyrimidino[5,4-d]pyrimidine; PE, phenylephrine chloride or (R)-(−)-1-(3-hydroxyphenyl)-2-methylaminomethyl hydrochloride; EFS, electrical field stimulation; ANOVA, analysis of variance; ENT, equilibrative nucleoside transporter; CNT, concentrative nucleoside transporter.
cleotidase to adenosine, a nucleoside base that acts as a local modulator and exerts a diverse range of physiological actions.

The actions of adenosine are mediated by four known subtypes of adenosine receptors, referred to as A1, A2A, A2B, and A3. All are members of the P1 family of purine receptors. Each adenosine receptor has a unique pharmacological profile, tissue distribution, and effector coupling. All four subtypes are members of the G protein-coupled receptor superfamily, and, because extracellular adenosine levels are quite variable (depending on the tissue and the degree of metabolic activity), their basal levels of stimulation vary enormously (Fredholm et al., 2001). Signaling via adenosine receptors is thought to occur mainly through inhibition or stimulation of adenylyl cyclase, although activation of other pathways, such as phospholipase C, calcium, arachidonic acid, and mitogen-activated protein kinases, have also been described previously (Jacobson and Gao, 2006). Whereas activation of the A1 and A3 adenosine receptors inhibits adenylyl cyclase activity and also results in increased activity of phospholipase C, activation of the A2A and A2B subtypes increases adenylyl cyclase activity. These effects are associated with activation of Gi and Gs proteins, respectively (Fredholm et al., 2001; Jacobson and Gao, 2006). In addition to activation of postjunctional receptors, adenosine can bind to prejunctional P1 receptors and modulate the release of excitatory neurotransmitters in both the peripheral and central nervous systems (Rongen et al., 1996; Duarte-Araújo et al., 2004; Burnstock, 2006).

Adenosine arrives in the interstitial fluid by direct release from cells (through a transporter or as a result of cell damage) or enzyme-mediated hydrolysis of extracellular adenine nucleotides, such as ATP, adenosine diphosphate (ADP), and adenosine monophosphate (AMP). Ectonucleotidases, present on the extracellular surface of many cells, rapidly shift signaling of adenine nucleotides (ATP, ADP, and AMP) to signaling through adenosine receptors. Ecto-5'-nucleotidase is the major enzyme responsible for the conversion of extracellular adenine nucleotides to adenosine (Zimmermann, 2000). Adenosine itself is rapidly metabolized by adenosine kinase to AMP, and, to a lesser degree, by adenosine deaminase to inosine. Adenosine can also be taken up by nerve terminals to allow resynthesis of adenine nucleotides, more specifically ATP (Zimmermann, 2000; Burnstock, 2006).

Little information exists on the role of adenosine as a neuromodulator of penile erection. Previous reports demonstrated that intracavernous injection of adenosine induces full erection in dogs (Takahashi et al., 1992) and that the erectile response induced by cavernous nerve stimulation is enhanced by the adenosine uptake inhibitor dipiridamole (Takahashi et al., 1991). In the rabbit corpus cavernosum, adenosine has potent relaxant activity, acting through the A2A receptor subtype (Mantelli et al., 1995) and it also modulates both adrenergic and NANC neurotransmission (Chiang et al., 1994). Adenosine A2 receptors were also identified in corpora cavernosa from human (Filippi et al., 2000) and dogs (Noto et al., 2001). In both tissues, adenosine induces NO-independent relaxation, and it has a direct relaxing effect on cavernous smooth muscle.

Based on the observations discussed above, we hypothesized that in murine corpora cavernosa, stimulation of sympathetic nerves enhances local adenosine production, which in turn exerts a direct relaxant effect on cavernosal smooth muscle cells and negatively modulates sympathetic neurotransmission. Since activation of adenosine receptors may be influenced not only by direct-acting ligands but also by agents that increase extracellular adenosine levels, such as inhibitors of adenosine metabolism or adenosine cellular uptake (Jacobson and Gao, 2006), we have used various pharmacological tools to evaluate the effects of adenosine on contractile responses mediated by transmural sympathetic nerve stimulation as well as the direct effects of adenosine on murine cavernosal smooth muscle cells.

Materials and Methods

Animals. Thirty-two male C57BL/6 mice (16 weeks-old, 25–30 g; Harlan, Indianapolis, IN) were used in these studies. All procedures were performed in accordance with the Guiding Principles in the Care and Use of Animals, approved by the Medical College of Georgia Committee on the Use of Animals in Research and Education. The animals were housed four per cage on a 12-h light/dark cycle, and they were fed on a standard chow diet with water ad libitum.

Drugs and Solutions. Physiological salt solution of the following composition was used: 130 mM NaCl, 1.49 mM NaHCO3, 5.5 mM dextrose, 4.7 mM KCl, 1.17 mM MgSO4, 1.17 mM KH2PO4, 1.16 mM CaCl2·2H2O, and 0.026 mM EDTA. Acetylcholine chloride, atropine, N-nitro-L-arginine methyl ester (L-NAME), adenosine, 2-chloroadenosine, phenylephrine chloride, 5'-isotubercidin, erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA), C8031, and C101 were purchased from Sigma-Aldrich (St. Louis, MO). MRS1706, SCH5826, dilazep dihydrochloride, dipiridamole, and CV1808 were from Tocris Cookson Inc. (Ellisville, MO). All reagents used were of analytical grade. Stock solutions were prepared in deionized water, ethanol (2-chloroadenosine), or dimethyl sulfoxide (C8031, C101, MRS1706, SCH5826, dilazep, and CV1908), and they were stored in aliquots at −20°C; dilutions were made uniformly before use.

Functional Studies in Cavernosal Strips. After euthanasia, the penis was excised, transferred into ice-cold buffer, and dissected to remove the tunica albuginea, as described previously (Teixeira et al., 2005). One crural strip preparation (1 × 10 mm) was obtained from each corpus cavernosum (two crural strips from each penis). Cavernosal strips were mounted in 4-ml myograph chambers (Danish Myo Technology, Aarhus, Denmark) containing buffer at 37°C continuously bubbled with a mixture of 95% O2 and 5% CO2. The tissues were stretched to a resting force of 2.5 mN, and they were allowed to equilibrate for 60 min. Changes in isometric force were recorded using a PowerLab/S8P data acquisition system (Chart software, version 5.0; ADInstruments, Colorado Springs, CO). To verify the contractile ability of the preparations, a high KCl solution (120 mM) was added to the organ baths at the end of the equilibration period. Cumulative concentration-response curves to adenosine (10⁻⁸–3 × 10⁻⁴ M), 2-chloroadenosine (10⁻⁸–10⁻³ M), and CV1808 (10⁻⁸–3 × 10⁻⁴ M) were obtained in cavernosal strips contracted with 10 μM phenylephrine (PE). Electrical field stimulation (EFS) was applied to strips placed between platinum pin electrodes attached to a stimulus splitter unit (Stimu-Splitter II; Med-Lab Instruments, Loveland, CO), which was connected to a Grass S88 stimulator (Astro-Med, West Warwick, RI). EFS was conducted at 50 V, 1-ms pulse width, and trains of stimuli lasting 10 s at varying frequencies (1–32 Hz). When antagonists or inhibitors were used, drugs were incubated during 30 to 45 min before concentration-response or frequency-response curves were performed. Time control experiments were performed to determine force development of cavernosal strips not related to the effects of each antagonist/inhibitor. Control solutions containing vehicle levels of ethanol and dimethyl sulfoxide were also used through the experimental protocols.
**Statistical Analysis.** Contractions were recorded as changes in the displacement (millinewtons) from baseline, and they were normalized by the dry weight of cavernous strips (milligrams). Data are represented as millinewtons per microgram of tissue for n experiments. Relaxation was expressed as percentage change from the PE-contracted levels. Agonist concentration-response curves were fitted using a nonlinear interactive fitting program (Graph Pad Prism 3.0; GraphPad Software Inc., San Diego, CA). Agonist potencies and maximum response are expressed as negative logarithm of the molar concentration of agonist producing 50% of the maximum response (pD2) and maximum effect elicited by the agonist (Emax), respectively. Concentration ratios were determined from EC50 values in the presence and absence of the antagonists. The concentration-response curves to the agonist in the presence or absence of the antagonists were analyzed by plotting the negative logarithm of the ratio of concentrations of the agonist that produced the same effect (50% contraction) in the presence and absence of the antagonist minus 1 [log (concentration ratio – 1)] against the negative logarithm of the concentration of antagonists (i.e., Schild plot analysis). The intercept on the abscissa yields the negative logarithm of the concentration of antagonist that induces a 2-fold rightward shift of the concentration-response curve to the agonist (pA2). Statistically significant differences were calculated by one-way analysis of variance (ANOVA) or Student’s t test. p < 0.05 was considered as statistically significant.

**Results**

The weight of the male C57BL/6 mice used in these studies was 27.9 ± 0.3 g (n = 36), and the average dry weight of the cavernous strips was 1.9 ± 0.1 mg (n = 72). Stimulation with 120 mM KCl induced a contractile response of 368 ± 52 mN/μg (n = 32).

### Relaxing Effect of Adenosine and Analogs.

The addition of 10^-5 M PE to the bathing medium caused a submaximal contraction of mouse cavernosal segments, and it generated active force of 526 ± 47 mN/μg (n = 18), which consisted of a rapid rise in force followed by a slower rise to a sustained level within 10 min. The cumulative addition of adenosine (10^-5–3 × 10^-2 M; n = 8), 2-chloroadenosine (stable analog of adenosine; 10^-6–10^-3 M; n = 6), or CV1808 (A2A/A2B agonist; 10^-6–3 × 10^-4 M; n = 8) produced concentration-dependent relaxations of PE-contracted tissues (p < 0.05; Fig. 1), with pD2 values of 5.6 ± 0.5; 4.6 ± 0.3, and 4.4 ± 0.8, respectively (Table 1). Maximal responses elicited by 2-chloroadenosine (31.3 ± 5.1%) and CV1808 (25.1 ± 4.9%) were significantly augmented (p < 0.05) in comparison with those elicited by adenosine (13.0 ± 1.9%) (Fig. 1; Table 1).

### Effects of A2A and A2B Antagonists on 2-Chloroadenosine-Induced Relaxation.

To determine the receptor subtype(s) that mediate adenosine-induced relaxation of cavernosal strips, concentration-response curves to 2-chloroadenosine were performed in the absence (vehicle) or presence of different concentrations of SCH58261 (A2A antagonist; 10^-9–10^-6 M) or MRS1706 (A2B antagonist; 10^-8–10^-6 M). The more stable analog of adenosine, 2-chloroadenosine, was used in these experiments because concentration-dependent inhibitory effects of the antagonists would be easier to identify. SCH58261 (10^-9–10^-6 M) produced concentration-dependent rightward displacements of the 2-chloroadenosine concentration-response curves with reduction of the maximal response (Fig. 2A). Schild analysis yielded a pA2 value for SCH58261 of 7.67 ± 0.9 and a slope of 0.37 ± 0.19, which was significantly different from unity. Likewise, MRS1706 (10^-8–10^-6 M) displaced the curves for 2-chloroadenosine to the right (Fig. 2B). Schild analysis yielded a pA2 value for MRS1706 of 6.97 ± 0.8 and a slope of 0.7 ± 0.3, which was significantly different from unity. The combination of 10^-7 M SCH58261 and 10^-7 M MRS1706 resulted in a greater antagonism than that exerted by the antagonists when added individually, and it almost completely abrogated 2-chloroadenosine-induced relaxation. Addition of both SCH58261 and MRS1706 completely inhibited adenosine-induced relaxation. The pD2 value of 2-chloroadenosine concentration-response curves as well as the Emax values observed in the absence and presence of SCH58261 and MRS1706 are given in Table 2.

### Effects of Inhibitors of Adenosine Metabolism or Uptake on EFS-Induced Contraction.

In cavernosal smooth muscle strips under resting conditions, EFS (1-32 Hz) produced frequency-dependent contractions that were virtually abolished by the sympathetic nerve blocking agent bretylium tosylate (3 × 10^-5 M; n = 4) and by the α-adrenergic antagonist terazosin (10^-6 M; n = 3), confirming that these responses are neuronal in origin and adrenergic in nature (Fig. 3). Addition of atropine (a muscarinic receptor antagonist; 10^-6 M) plus L-NAME (nonselective inhibitor of nitric-oxide synthase; 10^-4 M) caused augmentation of EFS-induced contractions, as shown at Fig. 3.

To evaluate the effects of endogenous adenosine on the contractions induced by EFS of sympathetic nerves, the following compounds, which are known to increase adenosine levels, were used: 5'-iodotubercidin (adenosine kinase inhibitor; Davies et al., 1986; 10^-7–2 × 10^-5 M), EHNA (adenosine deaminase inhibitor; Ullman et al., 1976; 10^-7–10^-4 M), and dipyridamole (inhibitor of adenosine transport; Kla-

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**Table 1**

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<tr>
<th>Agonist</th>
<th>Emax (%)</th>
<th>pD2</th>
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<tr>
<td>Adenosine</td>
<td>13.0 ± 1.9</td>
<td>5.66 ± 0.5</td>
</tr>
<tr>
<td>2-Chloroadenosine</td>
<td>31.3 ± 5.1*</td>
<td>4.63 ± 0.3</td>
</tr>
<tr>
<td>CV1808</td>
<td>25.1 ± 4.9*</td>
<td>4.44 ± 0.8</td>
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* p < 0.05 vs. adenosine (ANOVA).

**Fig. 1.** Effects of adenosine, 2-chloroadenosine, and CV1808 (A2A/A2B agonist) in murine cavernosal strips contracted with 10^-5 M PE. Experimental values of the relaxations induced by adenosine (■, n = 8), 2-chloroadenosine (○, n = 6), and CV1808 (□, n = 8) were calculated relative to the maximal changes from the contraction produced by PE in each tissue, which was taken as 100%. Data represent the mean ± S.E.M. of n experiments. * p < 0.05 versus adenosine.
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**Fig. 2.** Effects of A2A and A2B antagonists on 2-chloroadenosine-induced relaxation in murine cavernosal strips contracted with 10^{-5} M PE. Experimental values of the relaxations induced by 2-chloroadenosine in the absence (n = 7) or presence of SCH58261 (A2A antagonist; 10^{-2}–10^{-6} M; n = 4 for each antagonist concentration) (A) or MRS1706 (A2B antagonist; 10^{-6}–10^{-6} M; n = 4 for each antagonist concentration) (B) were calculated relatively to the maximal changes from the contraction produced by PE in each tissue, which was taken as 100%. Insets, Schild plot analysis. Data represent the mean ± S.E.M. of n experiments.

**Table 2**

<table>
<thead>
<tr>
<th>Group</th>
<th>E_{max} (%)</th>
<th>pD_{2}</th>
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<tbody>
<tr>
<td>Vehicle</td>
<td>28.2 ± 4.3</td>
<td>5.23 ± 0.05</td>
</tr>
<tr>
<td>SCH58261 (10^{-9} M)</td>
<td>20.5 ± 4.2</td>
<td>5.36 ± 0.05</td>
</tr>
<tr>
<td>SCH58261 (10^{-8} M)</td>
<td>13.7 ± 4.5*</td>
<td>4.79 ± 0.10</td>
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<tr>
<td>SCH58261 (10^{-7} M)</td>
<td>12.0 ± 4.0*</td>
<td>5.04 ± 0.17</td>
</tr>
<tr>
<td>SCH58261 (10^{-6} M)</td>
<td>13.1 ± 4.5*</td>
<td>4.38 ± 0.27</td>
</tr>
<tr>
<td>MRS1706 (10^{-9} M)</td>
<td>26.0 ± 3.4</td>
<td>5.12 ± 0.03</td>
</tr>
<tr>
<td>MRS1706 (10^{-8} M)</td>
<td>15.3 ± 5.7*</td>
<td>5.06 ± 0.12</td>
</tr>
<tr>
<td>MRS1706 (10^{-7} M)</td>
<td>14.0 ± 5.5*</td>
<td>5.19 ± 0.13</td>
</tr>
<tr>
<td>SCH58261 (10^{-7} M) +</td>
<td>5.04 ± 1.04*</td>
<td>N.D.</td>
</tr>
<tr>
<td>MRS1706 (10^{-7} M)</td>
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N.D., not determined. *p < 0.05 vs. control (2-chloroadenosine) (ANOVA).

To determine whether 5'-iodotubercidin and dipyridamole would change adenosine-mediated effects via A2A/A2B postsynaptic receptors, we performed concentration-response curves to adenosine in the absence and in the presence of 10^{-6} M 5'-iodotubercidin and 10^{-5} M dipyridamole (Fig. 5B). The cumulative addition of adenosine (10^{-6}–3 × 10^{-4} M) produced concentration-dependent relaxations that were not different among the three groups: pD_{2} and E_{max} were as follows, respectively: adenosine + vehicle, 6.11 ± 0.09 and 11.5 ± 1.7%; adenosine + 5'-iodotubercidin, 6.02 ± 0.08 and 14.6 ± 4.2%; and adenosine + dipyridamole = 5.83 ± 0.06 and 12.4 ± 4.1%. n = 4.

Because adenosine has been shown to inhibit norepinephrine release from sympathetic nerve endings in a variety of in vitro models and because adenosine A1 receptors are thought to be involved in this inhibitory effect (De Mey et al., 1979; Illes et al., 1988; von Kugelgen et al., 1992; Grimm et al., 2001; Queiroz et al., 2003; Burnstock, 2006), we evaluated the effects of the adenosine A1 receptor agonist C8031 (10^{-9}–10^{-6} M; Klotz et al., 1989) on contractile responses induced by EFS in murine cavernosal strips (Fig. 6A). The A1 agonist right-shifted the contractile responses to EFS, with a significant inhibitory effect at the dose of 10^{-6} M (p < 0.05). At 4 Hz, contractile responses (millinewtons per microgram of tissue) in cavernosal strips were vehicle, 234 ± 43, n = 6; 10^{-6} M C8031, 248 ± 13, n = 4; 10^{-7} M C8031, 193 ± 35, n = 4; and 10^{-6} M C8031, 129 ± 38, n = 3. The A1 agonist at the dose of 10^{-6} M had no relaxant effects when tested directly on 10^{-5} M PE-contracted cavernosal strips (C8031: 4.7 ± 3.3% relaxation, n = 4 versus vehicle 6.9 ± 5.4% relaxation, n = 3). Furthermore, contractile responses to EFS were enhanced in the presence of the adenosine A1 receptor antagonist C101 at 10^{-7} M (Bruns et al., 1987), and the inhibitory effects of 10^{-6} M 5'-iodotubercidin were not observed in the presence of 10^{-7} M C101 (Fig. 6B). Furthermore, the A1 receptor antagonist C101 at 10^{-7} M did not change adenosine- or 2-chloroadenosine-induced relaxation of cavernosal strips.

**Discussion**

The present study shows that adenosine has direct relaxant effects mediated by the activation of A2A and A2B receptor subtypes, in murine corpora cavernosa. In addition,
agents known to increase adenosine levels, such as 5'-iodotubercidin (adenosine kinase inhibitor), EHNA (adenosine deaminase inhibitor), and dipyridamole (inhibitor of adenosine transport) in the frequency-response curves elicited by EFS (1–32 Hz) in murine corpus cavernosum. After completion of a control curve to EFS, the tissues were incubated in the presence of 5'-iodotubercidin (10^{-7}–2 \times 10^{-5} M; n = 4 for each concentration of the inhibitor) (A), EHNA (10^{-7}–10^{-5} M; n = 4) (B), or dipyridamole (10^{-7}–2 \times 10^{-5} M; n = 4) (C), and a second curve to EFS was performed. Experimental values of contraction were calculated relative to the dry weight of cavernosal strips and are represented as millinewtons per microgram of tissue. Data represent the mean ± S.E.M. of n experiments. *, p < 0.05 compared with control values.

The first set of experiments, we observed that murine cavernosal strips display relaxant responses to adenosine, 2-chloroadenosine, and the A_{2A}/A_{2B} receptor agonist CV1808. Relaxant responses to 2-chloroadenosine and CV1808 were greater than those induced by adenosine, consistent with a previous study reporting that 2-chloroadenosine and CV1808 are more potent than adenosine, in both biochemical (receptor binding) and functional (inhibition of neurogenic contractions of the vas deferens) assays (Taylor and Williams, 1982). The greater relaxant responses to 2-chloroadenosine and CV1808, in comparison with that of adenosine, may indicate that adenosine and its analogs undergo differential metabolism or cellular uptake processes.

The high apparent potency of 2-chloroadenosine compared with adenosine has been related to the former being neither taken up by nucleoside transporters nor deaminated, whereas the apparent potency of adenosine would be masked by uptake and deamination in the tissue (Muller and Paton, 1979). However, 2-chloroadenosine does undergo cellular uptake in certain cell types (Stolk et al., 2005; Minelli et al., 2006). Furthermore, it is known that mammalian cells exhibit different isoforms of equilibrative (ENT1, ENT2, ENT3) and concentrative nucleoside transporters (CNT1, CNT2, CNT3), and that although the isoforms possess some overlap in transport of several adenosine analogs, they also exhibit distinct differences in capacity to interact with some adenosine receptor ligands and adenosine-based drugs (King et al., 2006). Likewise, several enzyme families (ectonucleoside triphosphate diphosphohydrolase, ectonucleotide pyrophosphatase/phosphodiesterase, alkaline phosphatases, and ecto-5'-nucleotidase), with overlapping substrate specificities and tissue distributions, are involved in the metabolism of adenosine nucleoside, adenosine nucleotides (ATP, ADP, and AMP), and a variety of diadenosine polyphosphates (Zimmermann, 2000). To our knowledge, the proteins that mediate...
Adenosine transport and metabolism in penile tissue have not been characterized, and our laboratory is currently addressing these aspects. One should still consider the possibility that adenosine may also act on receptors that mediate contractile responses. So far, we have observed that neither adenosine, 2-chloroadenosine, CV1808, nor the adenosine A1 receptor agonist C8031 induces contractile responses in cavernosal strips (data not shown).

That both the selective A2A (SCH58261) and A2B (MRS1706) antagonists inhibited 2-chloroadenosine- as well as adenosine-mediated relaxation indicates that both the A2A and A2B receptor subtypes mediate the relaxant actions of adenosine in murine corpora cavernosa. Previous reports demonstrated that adenosine has potent relaxant activity on the rabbit corpus cavernosum, acting through the A2A receptor subtype (Mantelli et al., 1995). Adenosine A2 receptors were also identified in human corpora cavernosa, where adenosine also induces NO-independent relaxation (Filippi et al., 2000). Interestingly, in vivo, adenosine increased human cavernosal peak blood flow velocity in a time- and dosedependent manner, but it does not induce penile erection. The authors mentioned that the lack of effects of adenosine on penile tumescence may be related to the ability of adenosine to also induce relaxation of human deep dorsal penile veins (Filippi et al., 2000).

In the second set of experiments, we evaluated whether agents that increase adenosine levels, such as 5'-iodotubercidin (adenosine kinase inhibitor; Davies et al., 1986), EHNA (adenosine deaminase inhibitor; Ullman et al., 1976), and dipyridamole (inhibitor of adenosine transport; Klabunde, 1983; Kiss et al., 2000), would interfere with cavernosal contractile responses induced by electrical stimulation of sympathetic nerves. Because these compounds inhibited murine cavernosal contractile responses generated by EFS, but they did not change direct responses to adenosine, we hypothesized that inhibition of sympathetic-mediated responses are due to prejunctional effects of adenosine.

Many reports have shown that adenosine modulates norepinephrine release from sympathetic nerve endings (Burnstock, 2006). Most commonly, the adenosine A1 receptor subtype negatively modulates norepinephrine release, whereas the A2 receptor subtypes enhance neurotransmitter release (von Kugelgen et al., 1992; Rongen et al., 1996; Burnstock, 2006). Considering that adenosine A1 prejunctional inhibitory receptors were described in a variety of tissues—canine saphenous vein (De Mey et al., 1979), rabbit mesenteric arteries (Illes et al., 1988), rat heart (Grimm et al., 2001), and rat vas deferens (Queiroz et al., 2003)—we decided to evaluate the effects of an adenosine A1 receptor agonist on contractile responses induced by EFS in murine cavernosal strips. The A1 agonist C8031 right-shifted the contractile responses to EFS, supporting a role for A1 receptors on the inhibitory effects induced by 5'-iodotubercidin, EHNA, and dipyridamole. More interesting is the observation that contractile responses of murine cavernosal strips to EFS were enhanced in the presence of an adenosine A1 receptor antagonist and that the inhibitory effects of 5'-iodotubercidin were not observed when the A1 antagonist was present. Since we have not observed changes in adenosine-induced relaxation in the presence of these compounds, the possibility that the decreased contractile responses induced by EFS in the presence of 5'-iodotubercidin, dipyridamole, or EHNA are the result of postsynaptic activation of A2A/A2B receptors was discharged. Our results differ from those obtained by Mantelli et al. (1995), in isolated rabbit cavernosal smooth muscle, where adenosine-induced relaxation is potentiated by both the adenosine deaminase inhibitor EHNA and adenosine uptake inhibitor dipyridamole. One possible explanation would be that increased adenosine levels would preferentially activate receptors with a higher affinity for adenosine, which is the case for the A1 receptor subtype (Fredholm et al., 2001). Species variation regarding the actions of adenosine or adenosine receptor subtypes should also be taken into consideration. Together, these data suggest that adenosine negatively modulates sympathetic neurotransmission in murine corporal tissue.

Penile arteries and veins and cavernosal smooth muscle receive a rich adrenergic innervation, and it is generally accepted that the penis is kept in the flaccid state mainly via a tonic activity of the sympathetic nerves. The prejunctival actions of adenosine, negatively modulating contractile responses mediated by activation of sympathetic nerves, may prevent excessive adrenergic stimulation of the penis, which would make penile tumescence more difficult to occur, or which would be more difficult to overcome in the presence of an erection stimulus. Although our data suggest that there is a balance between the purinergic and sympathetic systems in murine corpus cavernosum, the exact role of adenosine in
penile erection physiology and pathophysiology remains to be established. The in vivo effects of adenosine receptor antagonists as well as those of drugs that enhance adenosine levels are being evaluated, and they will help to clarify the physiological role of adenosine in mouse penile physiology. If adenosine does play a role in penile erection, drugs that increase adenosine levels, such as dipyridamole, which has already been used in human patients to prevent ischemic heart disease (due to its vasodilator and antiplatelet activation effects), may have therapeutic importance in the treatment of erectile dysfunction. However, the finding that purines, such as caffeine and theobromine, in concentrations as they occur in coffee, tea, and chocolate, as well as their synthetic derivatives such as theophylline, inhibit the vascular actions of dipyridamole (possibly as inhibitors of the adenosine A2 receptors) may cast some doubt on the therapeutic value of dipyridamole, especially because many soft drinks contain caffeine. Alternatively, many actions of caffeine and other methylxanthines are mediated via inhibition of cAMP-phosphodiesterase, and consequent increase in intracellular cAMP concentrations, which, in turn, facilitate smooth muscle relaxation and penile erection.

Adenosine is an endogenous nucleoside, and extracellular adenosine is required for P1 receptor activation. Two pathways have been identified for formation and cellular release of adenosine: the classical pathway, which relies on intracellular formation of adenosine from adenine nucleotides and cellular efflux of adenosine via ENTs; and the alternate pathway, which involves cellular release of adenine nucleotides, hydrolysis by ecto-5′-nucleotidases, and extracellular formation of adenosine (Zimmermann, 2000; Burnstock, 2006; King et al., 2006). We speculate that, in the penis, adenosine may be formed in the cavernosal smooth muscle cells, because this has already been demonstrated in vascular smooth cells (Dubey et al., 1996) and also because cavernosomal smooth muscle cells use cAMP as a second messenger (Andersson, 2001). Adenosine may also result from the sequential hydrolysis of ATP and other adenosine nucleotides, and ATP itself may be released from nerve terminals (either adrenergic, cholinergic, or NANC nerves) or by the mechanical distortion of smooth muscle and endothelial cells in response to tumesence. The effects of adenosine on cholinergic and NANC neurotransmission in the murine penis tissue are being investigated.

One may consider that the inhibitory effects of these compounds on transmural adrenergic nerve-induced contraction may be due to actions of ATP. In fact, ATP and other purines decrease both basal tension and phenylephrine-stimulated tension in isolated rabbit corpus cavernosum preparations (Tong et al., 1992; Wu et al., 1993). ATP acts as a potent and NO-independent relaxant agent of human and rabbit corpus cavernosum (Filippi et al., 1999). The effects of ATP are partially attributable to the metabolic breakdown of ATP into adenosine, but also they are also due to direct stimulation of P2 receptors. Human corporal cavernosal strips relax in response to stimulation of P2Y purinoceptors via NO release (Shalev et al., 1999). Furthermore, ATP was suggested as a NANC transmitter in the rabbit corporal cavernosal tissue, and purinergic transmission may be an important component involved in the initiation and maintenance of penile erection in this species (Tong et al., 1992).

Finally, another aspect to be considered is that the compounds tested may have actions other than those related to adenosine. Accordingly, adenosine-independent effects of dipyridamole (e.g., inhibition of NO production by activated polymorphonuclear neutrophils) have been described previously (Wykretowicz et al., 2004), but these effects were observed at higher concentrations than were used in this study. In summary, we have shown that adenosine has dual effects on murine corpora cavernosa. Adenosine directly relaxes cavernosal smooth muscle cells by the activation of A2A and A2B receptor subtypes, and adenosine also negatively modulates sympathetic neurotransmission by the activation of the A1 receptor subtype. These data suggest that adenosine may have a role in modulating the physiological mechanisms of erection in mice.


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