Enhanced Sensitivity of Heart Cells to Adenosine and Up-Regulation of Receptor Number After Treatment of Guinea Pigs With Theophylline

Sheng-Nan Wu, Joel Linden, Sergio Visentin, Michael Boykin, and Luiz Belardinelli

Experiments were carried out in hearts from guinea pigs that were fed either the adenosine receptor antagonist theophylline (0.6 mg/ml) or no drug. The $A_1$ adenosine receptor radioligand $[^{125}]$aminobenzyladenosine bound to a single affinity class of receptors in heart cell membranes from control animals with $B_{max}$ and $K_D$ of $18.3 \pm 1.0$ fmol/mg protein and $3.7 \pm 0.6$ nM, respectively ($n=8$). Heart cell membranes from animals fed theophylline for 2, 7, and 14 days bound the radioligand with about the same affinity, but the number of binding sites was significantly increased ($p<0.01$) to $30.6 \pm 1.7$ (n=3), $30.0 \pm 0.8$ (n=3), and $27.3 \pm 2.9$ (n=4), respectively. Nearly identical results were obtained with membranes prepared from enzymatically dispersed ventricular myocytes. Fourteen days of theophylline treatment also produced a small increase (12%, $p<0.01$) in the number of binding sites in membranes derived from cerebral cortices. Isolated ventricular myocytes prepared from animals fed no drug or theophylline for 7 days were used to determine the effect of adenosine on 20 nM isoproterenol-stimulated calcium current ($I_{Ca}$) measured by the whole-cell patch-clamp technique. Adenosine reduced isoproterenol-stimulated $I_{Ca}$ without affecting the activation or inactivation kinetics of the current; $I_{Ca}$ density was reduced less by 5 μM adenosine in cells from control (25±3 to 21±3 μA/μF) than in cells from theophylline-fed animals (26±5 to 17±2 μA/μF). Although a high concentration (0.5 mM) of adenosine abolished isoproterenol-stimulated $I_{Ca}$ in cells from control or theophylline-fed animals, the $IC_{50}$ for adenosine was sixfold less in cells derived from theophylline-fed animals than in cells from control animals (4.6±0.6 μM and 28.3±1.4 μM, respectively, $p<0.01$). In contrast, the increase in $I_{Ca}$ in response to isoproterenol alone and the potency of acetylcholine to antagonize this effect of isoproterenol were the same in both groups of cells. A maximally effective concentration of N-phenylisopropyladenosine (0.1 mM) inhibited isoproterenol-stimulated cyclic AMP accumulation less in cardiomyocytes from control than from theophylline-fed animals (28.7±1.8% vs. 42.0±4.2%, $p<0.05$). In summary, exposure of the myocardium to theophylline increases the number of adenosine receptors and the effects of receptor occupancy by agonists. These findings imply that the endogenous concentration of adenosine is high enough in the normoxic guinea pig heart to chronically maintain adenosine receptors in a partially down-regulated state. (Circulation Research 1989;65:1066-1077).

In isolated ventricular myocytes, adenosine has been shown to antagonize the electrophysiological actions of isoproterenol. That is, adenosine antagonizes the isoproterenol-induced increases in action potential duration, calcium conductance, delayed after-depolarizations, and sustained spontaneous activity. These effects are due to the ability of the nucleoside to bind to $A_1$ receptors on the extracellular surface of the sarcolemma and to inhibit adenylate cyclase activity. Although the density of $A_1$ adenosine receptors in the heart is 50-fold lower than in brain or adipose tissue, cardiac receptors can be measured even in crude heart membranes with the radioligand $[^{125}]$aminobenzyladenosine ($[^{125}]$ABA). This radioligand is an $A_1$ adenosine-selective agonist that exhibits an unusually high ratio of specific-to-nonspecific binding. Chronic (21 days) treatment of rats with theophylline has been shown to produce a 27.9% increase in
the number of $A_1$ adenosine receptors in rat brain cerebral cortex. 6 In a more recent study in which rats were treated twice daily for 14 consecutive days with theophylline, an increase in specific binding of $[^3H]$cyclohexyladenosine ($[^3H]$CHA) to membranes of cerebral cortex and cerebellum was associated with reduced susceptibility to convulsants. 7 However, the effects of chronic treatment with theophylline on $A_1$ adenosine receptors in heart have not yet been reported. In this study, we demonstrate a significant increase in the number of adenosine receptors in heart membranes of theophylline-fed animals. By comparison with cardiac myocytes from control animals, myocytes from theophylline-fed animals have an increased responsiveness to adenosine as an inhibitor of isoproterenol-stimulated inward calcium current ($I_{Ca}$) and cyclic AMP (cAMP) accumulation.

Materials and Methods

Chemicals

Isoproterenol, acetylcholine, atropine, theophylline, adenosine, collagenase (type II), dithiothreitol, benzamidine, phenylmethylsulfonyl fluoride, guanylylimidodiphosphate [Gpp(NH)p], and bovine serum albumin were purchased from Sigma Chemical (St. Louis, Missouri). R-phénylisopropyladenosine (RPIA), adenosine deaminase, and dispase were purchased from Boehringer Mannheim (Indianapolis, Indiana). Trypsin was purchased from Serva Fine Biochemicals (Heidelberg, FRG), Ro7-2955 (phosphodiesterase inhibitor) and dilazep (nucleoside transport inhibitor) were gifts of Dr. P.F. Sorter and Drs. A. Kaiser and P. Weber, respectively, of Hoffmann-La Roche (Nutley, New Jersey). l-Pindolol was a gift from Drs. H. Weidmann and H. Friedly of Sandoz Pharmaceuticals (East Hanover, New Jersey). The radioligand $[^125I]ABA$ was prepared as described by Linden et al. 5 $[^125I]$Pindolol was prepared by radioiodinating l-pindolol in the presence of chloramine T. $[^125I]$Pindolol was separated from pindolol by isocratic high-performance liquid chromatography (HPLC) over a 4.5 x 250 mm C18 column. The solvent was methanol/5 mM sodium phosphate, pH 6.0 (65:35). Elution times for pindolol and $[^125I]$pindolol were 10 and 24 minutes, respectively. $[^3H]$Quinuclidinyl benzilate ($[^3H]$QNB) was isolated by a technique similar to that described by Dendorfer et al. 8 In brief, guinea pig hearts were perfused for 10 minutes with an oxygenated (100% O2) prewarmed (35°C) modified Krebs-Henseleit solution containing (mM) NaCl 127, KCl 4.6, CaCl2 2, MgSO4 1.1, sodium pyruvate 2, glucose 8.3, creatine 10, ta urine 20, ribose 5, adenine 0.01, HEPES 5, and allopurinol 0.1, adjusted with NaOH to pH 7.4. The perfuse was then switched to a Ca2+-free solution for 10 minutes and subsequently to a protease solution containing 0.1 mg/ml dispase, 0.12 mg/ml trypsin, 0.32 mg/ml collagenase (type II), and 2.12 mg/ml albumin. After 20 minutes of perfusion with protease solution, the ventricles were removed, minced, and incubated for 5 minutes in the protease solution. Thereafter, cells were separated by mechanical agitation and filtered through 200 μm nylon mesh. The remaining undigested tissue was incubated for two additional 5-minute periods in the protease solution. The cells were harvested from the supernatant and stored at room temperature in Krebs-Henseleit solution containing 10 μM Ca2+. This procedure consistently yielded approximately 60–80% of rod-shaped calcium-tolerant ventricular myocytes.

Preparation and Protocols

Theophylline treatment of guinea pigs. Hartley guinea pigs (Hilltop Laboratories, Scottdale, Pennsylvania) of either sex and weighing 250–350 g were housed in individual cages with free access to food and water. Two groups of guinea pigs were fed drinking water that contained sucrose (20 g/l) and ascorbic acid (200 mg/l), either with or without theophylline (600 mg/l). The sucrose was added to offset the bitter taste of theophylline. On the day of experiments, plasma levels of theophylline were determined. Theophylline plasma levels in animals fed theophylline for 2, 7, and 14 days were 24.8±3.4 μg/ml (n=3), 22.2±2.3 μg/ml (n=7), and 23.0±4.2 μg/ml (n=5), respectively.

Membrane preparation. Crude membranes from cardiac ventricles or brain cerebral cortices were prepared identically, except that hearts were first perfused for 10 minutes with oxygenated Krebs-Henseleit solution at 8 ml/min to remove blood. In addition, in a separate series of experiments, cardiac membranes were prepared from enzymatically dispersed ventricular myocytes (see method below). After a rinse in ice-cold buffer A containing (mM) HEPES 10, EDTA 10, dithiothreitol 1, benzamidine 0.1, phenylmethylsulfonyl fluoride 10 (μg/ml), and 10% (wt/vol) sucrose at pH 7.4, ventricles, brain cerebral cortices, or ventricular myocytes were isolated and homogenized (polytron, setting 5, Brinkmann Instruments, Westbury, New York) for 10 seconds in 10 vol buffer A, filtered through four layers of gauze, and centrifuged at 20,000g for 30 minutes. The supernatant and bottom brown-colored layer of the pellet were discarded. The upper layer of the pellet was resuspended in 10 vol buffer B (buffer A minus sucrose and phenylmethylsulfonyl fluoride, with 0.1 mM EDTA and 2.5 mM MgCl2) and washed four times by centrifugation to ensure complete removal of theophylline. The final pellet was resuspended in 3 ml (ventricle) or 30 ml (cortex) buffer B and frozen at −70°C.

Isolated myocytes. Ventricular myocytes were isolated by a technique similar to that described by Dendorfer et al. 8 In brief, guinea pig hearts were perfused for 10 minutes with an oxygenated (100% O2) prewarmed (35°C) modified Krebs-Henseleit solution containing (mM) NaCl 127, KCl 4.6, CaCl2 2, MgSO4 1.1, sodium pyruvate 2, glucose 8.3, creatine 10, ta urine 20, ribose 5, adenine 0.01, HEPES 5, and allopurinol 0.1, adjusted with NaOH to pH 7.4. The perfuse was then switched to a Ca2+-free solution for 10 minutes and subsequently to a protease solution containing 0.1 mg/ml dispase, 0.12 mg/ml trypsin, 0.32 mg/ml collagenase (type II), and 2.12 mg/ml albumin. After 20 minutes of perfusion with protease solution, the ventricles were removed, minced, and incubated for 5 minutes in the protease solution. Thereafter, cells were separated by mechanical agitation and filtered through 200 μm nylon mesh. The remaining undigested tissue was incubated for two additional 5-minute periods in the protease solution. The cells were harvested from the supernatant and stored at room temperature in Krebs-Henseleit solution containing 100 μM Ca2+. This procedure consistently yielded approximately 60–80% of rod-shaped calcium-tolerant ventricular myocytes.

Radioligand binding assays. Binding assays were conducted as described in previous studies in which it has been demonstrated that $[^125I]$ABA binds with high affinity and specificity to $A_1$ adenosine recep-
tors of cardiac muscle and brain. Membranes were incubated with radioligand and 1.25 units/ml adenosine deaminase for 2 hours at 21°C. [125I]ABA synthesized as previously described was shown to coelute by HPLC with its nonradioactive counterpart, which was characterized by proton nuclear magnetic resonance and elemental analysis. For equilibrium binding assays, a fixed concentration of radioligand, 0.15–0.3 nM, was diluted with nonradioactive iodo ABA to achieve final concentrations ranging between 0.5 and 20 nM. Nonspecific binding, defined as ligand not displaced by 10 μM R-PIA, was determined and subtracted from the total binding to give specific binding. Nonspecific binding was nearly identical in membranes derived from control or theophylline-fed animals. To assess the effects of feeding animals with theophylline, tissues from groups of control and theophylline-fed guinea pigs were processed in parallel. The resultant membranes were used for radioligand binding assays conducted on the same day, with the same radioligand synthesized within 1 week of the experiment. Coupling of receptors to G proteins was assessed by examining the effects of Gpp(NH)p on agonist radioligand binding.

**Electrophysiological measurements.** Isolated ventricular myocytes were transferred into a recording chamber, which was mounted on the stage of an inverted microscope coupled to a TV camera system. Cells were continuously superfused at a rate of 2–4 ml/min with Krebs-Henseleit solution containing 2 mM CaCl2. The bath temperature in all experiments was maintained at 35 ± 1°C.

Membrane potentials and currents were recorded with “patch electrodes” (Kimax glass pipettes, Kimble Glass, Vineland, New Jersey) in a whole-cell mode. The electrodes had a tip diameter of 2–4 μm and resistances of 1–3 MΩ when filled with the following solution (mM): potassium chloride 10, potassium aspartate 130, sodium ATP 4, MgCl2 1, HEPES 10, and sodium EGTA 1, adjusted with KOH to pH 7.4. The resting potential and action potential duration at 90% repolarization (APD90) were measured. Voltage-clamp experiments were carried out with the single microelectrode technique. To correct for junctional potential, current was zeroed with the electrode in the bath solution but before contact with the surface of the myocyte. To inactivate the sodium current, the clamp pulses started from a holding potential that was set at values between -35 and -45 mV. To measure I

\[ I_{\text{Ca}} \]

clamp pulses of 150-msec duration from -40 mV to various potentials were applied. These depolarizing voltage-step command signals were generated at a rate of 0.3 Hz. The voltage drop across the electrode resistance caused by the current flow was electronically compensated. \[ I_{\text{Ca}} \] was normalized based on capacitive transient. Cell capacitance was determined at the beginning of the experiment from the capacitive transient from a ± 10 mV voltage-step pulse from resting membrane potential.

**cAMP determinations.** Ventricular myocytes were prepared as described above. freshly isolated cells were then preincubated in Krebs-Ringer-HEPES buffer containing 1 unit/ml adenosine deaminase, 0.5 nM Ro7-2956 (a phosphodiesterase inhibitor), and various concentrations of R-PIA for 10 minutes at 37°C. L-Isoproterenol (1 μM) and 0.5 mM ascorbic acid were added for 90 seconds, and then the
cells were lysed by the addition of 50 mM hydrogen chloride. After centrifugation at 10,000g for 30 minutes, cAMP in the supernatants was acetylated and quantitated by automated radioimmunoassay.16,17

Data recording and analyses. Binding data were fitted by the method of Scatchard18 or Marquardt19 to equations for one to three binding sites:

\[
B = \sum_{i=1}^{n} B_{\text{max}}[L]/(K_{d,i} + [L])
\]

where B is radioligand specifically bound and [L] is the free concentration of radioligand. To determine if radioligand binding parameters to membranes derived from control and experimental groups differed significantly, the method suggested by Motulsky and Ransnas was used.20 Differences in radioligand binding parameters were considered different if F tests evaluated at \( p < 0.01 \) indicated significant improvement in the goodness of fit (as determined from the sum of squares of the difference between the data and the fit curve) when two data sets were evaluated separately compared with the goodness of fit to a single curve when the data were pooled.20

Electrophysiological data consisting of voltage and current tracings were displayed on a storage oscilloscope (model 5113, Tektronix, Beaverton, Oregon) and on a two-channel strip-chart recorder (Brush model 2200S, Gould, Cleveland, Ohio). The data were digitized at the sampling frequency of 5 kHz on line with a 2801-A board (Data Translation, Marlborough, Massachusetts) and stored on hard disc in an AT&T 6300 microcomputer. Subsequent analyses were conducted off line by replaying the data into the microcomputer, which was programmed to emulate a digital oscilloscope with graphics capability.21

Ic, was defined and analyzed as the "visual estimate"; that is, the peak net current was subtracted from the current at the end of the clamp step.22,23 The time course of inactivation of Ic, was determined according to the two-exponential model described by Isenberg and Klockner.22 By use of a least-squares fit iteration, the steady-state current was subtracted from the net current. The data were logarithmically rectified and then fit to the two-exponential model by linear regression analysis. To normalize variations in peak Ic, the effects of isoproterenol and adenosine on Ic, of cardiac myocytes from control and theophylline-fed guinea pigs were normalized by integration of capacitive transient; that is, Ic, density was determined according to peak Ic divided by cell capacitance (\( \mu A/\mu F \)). Because of cell-to-cell variations in the response to isoproterenol, the percent inhibition of the current caused by adenosine was calculated as follows:

\[
\% \text{ of inhibition} = \left( \frac{I_{c,\text{ISO}} - I_{c,\text{ISO+drug}}}{I_{c,\text{ISO}} - I_{c,\text{control}}} \right) \times 100
\]

where Ic,ISO and Ic,ISO+drug are peak Ic, in the presence of 20 nM isoproterenol without and with given concentrations of drug, that is, adenosine or acetylcholine, respectively. IC50 values of adenosine, that is, the doses required to inhibit isoproterenol-stimulated Ic, by 50%, were calculated by Wilkinson's computational procedure for nonlinear regression analysis.24 Because the increase in cAMP in response to isoproterenol varied in magnitude among individual batches of heart cells, the effect of R-PIA was also calculated as percent inhibition of the isoproterenol-induced increase.

All values are reported as mean ± SEM. Student's unpaired t test was used for the statistical analyses of electrophysiological and cAMP data. Differences between values were considered significant when \( p < 0.05 \).

Results

Theophylline Pretreatment Increases the Number of Adenosine Receptors in Cardiac Membranes

The binding of \[^{125}I\]ABA was investigated in crude membranes from whole brains, ventricles, and enzymatically dispersed ventricular myocytes. As shown in Figure 1, crude membranes assayed in the presence of Mg2+ exhibited only a single high-affinity site for the radioligand. The addition of 0.1 mM Gpp(NH)p reduced specific binding of agonist radioligand to less than 10% of control; this occur-

### TABLE 1. Effect of Long-term Treatment of Guinea Pigs With Theophylline on the Binding of Various Radioligands to Heart Membranes

<table>
<thead>
<tr>
<th>Target receptor</th>
<th>Radioligand</th>
<th>Concentration (pM)</th>
<th>Control (n=6–9)</th>
<th>Theo (n=4–7)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>A&lt;sub&gt;1&lt;/sub&gt; Adenosine</td>
<td>[^{125}I]ABA</td>
<td>450</td>
<td>4.6±0.5</td>
<td>8.2±0.8</td>
<td>0.01</td>
</tr>
<tr>
<td>Nucleoside transporter</td>
<td>[^{3}H]NBMPR</td>
<td>200</td>
<td>8.2±1.0</td>
<td>7.4±0.7</td>
<td>NS</td>
</tr>
<tr>
<td>β-Adrenergic</td>
<td>[^{125}I]indolol</td>
<td>220</td>
<td>15±3</td>
<td>15±2</td>
<td>NS</td>
</tr>
<tr>
<td>Muscarinic cholinergic</td>
<td>[^{3}H]QNB</td>
<td>3,330</td>
<td>137±14</td>
<td>147±16</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. Cardiac membranes were prepared from guinea pigs fed with theophylline (Theo) or vehicle (control) for 7–14 days as described in "Materials and Methods." \[^{125}I\]ABA, \[^{12}I\]laminobenzyladenosine; \[^{3}H\]NBMPR, \[^{3}H\]nitrobenzylthionine; \[^{3}H\]QNB, \[^{3}H\]quinucilindyl benzilate; NS, not significant. Nonspecific binding of the radioligands was evaluated by the addition of the following compounds: A<sub>1</sub> adenosine receptor, R-phenylisopropyladenosine; nucleoside transporter, 1.5 mM dilazep; β-adrenergic, 1 μM I-propranolol; muscarinic cholinergic, 1 μM atropine. \[^{3}H\]NBMPR and \[^{3}H\]QNB are specific radioligands that label nucleoside transporters and muscarinic cholinergic receptors, respectively.
rence suggests that this pool of receptors is coupled to G proteins and presumably to physiological responses of the heart mediated by A1 adenosine receptors. Specific binding of [125I]ABA to receptors measured in the presence of 0.1 mM Gpp(NH)p was too low to be accurately quantitated.

To examine the effects of theophylline on the properties of A1 adenosine receptors, nucleoside transport sites, β-adrenergic receptors, or cholinergic muscarinic receptors, binding sites on heart membranes from control animals or animals treated with theophylline for 14 days were initially investigated using single concentrations of the appropriate radioligands. As shown in Table 1, [125I]ABA binding was significantly increased in the membranes of hearts derived from theophylline-fed animals. In contrast, the specific binding of the nucleoside transporter NBMPR was not affected by the theophylline (Table 1). Likewise, the specific binding of [125I]pindolol and [3H]QNB to β-adrenergic and cholinergic muscarinic receptors, respectively, were not significantly different between the two groups. Note that there were more binding sites for [125I]ABA in brain (Figure 1A) than in heart (Figure 1B) membranes. More detailed analyses revealed that the number of binding sites for [125I]ABA in heart and brain membranes were significantly increased after 14 days of theophylline treatment, whereas there was no difference in the affinity of [125I]ABA for these sites (Figures 1 and 2A). Furthermore, there were also significant increases of similar magnitude in the number of [125I]ABA binding sites in heart membranes prepared from animals fed theophylline for 2 and 7 days (Figure 2B). Similar results were obtained if membranes were prepared from enzymatically dissociated ventricular myocytes. The number of binding sites for [125I]ABA was greater in the theophylline-treated group than in the control group, but there was no difference in $K_D$ values (Figure 3).

Theophylline Pretreatment Increases the Sensitivity of Cardiac Myocytes to Adenosine and R-PIA

To determine if the increase in [125I]ABA binding sites was associated with enhanced sensitivity of heart cells to adenosine, we investigated the inhibitory effect of adenosine and R-PIA on isoproterenol-stimulated $I_{Ca}$ and cAMP accumulation, respectively.

In this series of experiments, we used only myocytes that were quiescent and had well-defined striations and a rod-like shape. Myocytes with action potential duration (measured at 90% repolarization, $APD_{90}$) shorter than 80 msec or longer than 300 msec at 37°C or myocytes that responded to 20 nM isoproterenol with less than a 20% increase in $I_{Ca}$ were not used. Of all cells that fulfilled the above criteria, the resting membrane potential was $-82±1$ mV ($n=46$). There was no significant difference in resting potential between the two groups (control and theophylline-treated); hence, the above value represents the mean±SEM of the combined data from both groups. Likewise, the $APD_{90}$ in the control group was $157±2$ msec.

![Figure 2](http://circres.ahajournals.org/doi/abs/10.1161/01.RES.65.4.1070)

**Figure 2.** Graphs depicting [125I]-aminobenzyladenosine ([125I]-ABA) binding to ventricle membranes from animals fed no drug (control, □) or theophylline for 2 (●), 7 (○), or 14 (▲) days. In each of the two groups (control and theophylline-fed), the radioligand binding data were pooled from three to five ventricles processed together and assayed simultaneously with the same radioligand. Binding parameters were fit to the pooled data. In all cases, the control and theophylline data were fit significantly better by two curves than by one ($p<0.01$, see "Materials and Methods"). Panel A: Data for control and 14-day theophylline-fed groups. $B_{max}$ and $K_D$, respectively, are $16.3±1.0$ fmol/mg protein and $3.0±0.7$ nM for the control group and $27.3±2.9$ fmol/mg protein ($p<0.001$) and $2.5±0.9$ nM for the 14-day theophylline-fed group. Panel B: Data for control, 2-, and 7-day theophylline-fed (n=3) groups. $B_{max}$ and $K_D$, respectively, are $21.7±1.0$ fmol/mg protein and $4.8±0.6$ nM for the control group, $30.6±1.7$ fmol/mg protein ($p<0.001$) and $5.1±0.8$ nM for the 2-day theophylline-fed group, and $30.0±0.8$ fmol/mg protein ($p<0.001$) and $4.0±0.3$ nM for the 7-day theophylline-fed group. Pooled binding data from control membranes (panels A and B, n=8) gave $B_{max}$ and $K_D$ of $18.3±1.0$ fmol/mg protein and $3.7±0.6$ nM, respectively.
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**Figure 3.** Graph depicting <sup>125</sup>I-aminobenzyladenosine (<sup>125</sup>I-ABA) binding to cardiac membranes prepared from enzymatically dispersed ventricular myocytes of guinea pigs fed no drug (control, □, n=5) or theophylline for 7 days (●, n=6). In each of the two groups (control and theophylline), the radioligand binding data were pooled. B<sub>max</sub> and K<sub>D</sub>, respectively, are 21.5±2.3 fmol/mg protein and 4.9±1.5 nM for the control group and 30.3±3.1 fmol/mg protein and 6.9±1.8 nM for the theophylline-fed group. The K<sub>D</sub> values of the two groups were not significantly different (p>0.05), whereas B<sub>max</sub> in the theophylline-fed group was greater (p<0.01) than in the control group. The data were fit significantly better by two curves than by one (p<0.01, see "Materials and Methods").

(n=7), whereas in the theophylline-treated group it was 151±4 msec (n=6); the values are not significantly different.

Isoproterenol increased both action potential duration (Figure 4) and peak I<sub>Ca</sub> (Figure 5, upper panels). Isoproterenol (20 nM) prolonged APD<sub>90</sub> by an equivalent magnitude in cells derived from control and theophylline-treated guinea pigs (32±4 [n=7] vs. 34±3% [n=6], respectively; p>0.05). However, in the presence of isoproterenol, adenosine (5 µM) shortened the APD<sub>90</sub> significantly (p<0.05) less in cells from control than theophylline-treated animals (6±1% vs. 13±4%, respectively). A typical example is shown in Figure 4. The effects of isoproterenol and adenosine on peak I<sub>Ca</sub> were expressed in units of I<sub>Ca</sub> density (µA/µF) to minimize variability among cells. The data are summarized in Figure 6. There was no difference between the two groups of cells in the magnitude of the increase in I<sub>Ca</sub> density caused by isoproterenol. However, 5 µM adenosine inhibited the isoproterenol-stimulated I<sub>Ca</sub> significantly more in cells from theophylline-fed than in cells from control animals (Figure 6). The upper panels of Figure 5 depict an example of the effect of isoproterenol and adenosine on I<sub>Ca</sub>. These data indicate that the sensitivity of I<sub>Ca</sub> (enhanced by isoproterenol) to 5 µM adenosine is markedly increased by treating animals with theophylline. To determine whether the difference in the effect of adenosine to inhibit isoproterenol-stimulated I<sub>Ca</sub> also occurred at other membrane potentials, I<sub>Ca</sub> was examined at potentials between −40 and +50 mV. By comparing the two typical current-voltage curves shown in the lower panels of Figure 5, it can be seen that the threshold potential (around −35 mV) as well as the potential of maximum peak I<sub>Ca</sub> (+10 mV) were essentially the same in ventricular myocytes from control (panel B) and theophylline-fed (panel D) guinea pigs.

The calcium current tracings shown in the upper panels of Figure 5 were plotted semilogarithmically in Figure 7. As expected from a previous study, the inactivation time course of I<sub>Ca</sub> could be fitted by the sum of two exponentials, that is, a slow and a fast component. Isoproterenol (20 nM) increased peak I<sub>Ca</sub> without modifying its kinetics of inactivation; that is, the time constants of the slow and fast exponential components of I<sub>Ca</sub> did not differ significantly between

**Figure 4.** Tracings showing effects of isoproterenol and adenosine on the action potentials of two isolated ventricular myocytes with similar initial action potential durations from animals fed no drug (panel A) or theophylline (panel B) for 7 days. 1, control action potential; 2, action potential in the presence of 20 nM isoproterenol alone; 3, action potential in the presence of 20 nM isoproterenol with 5 µM adenosine. Action potential durations at 90% repolarization for 1-3, respectively, were 152, 200, and 193 msec for controls and 174, 204, and 175 msec for the theophylline-fed group.
the two groups. Furthermore, in the presence of isoproterenol, the addition of adenosine attenuated peak $I_{Ca}$ without affecting the inactivation time constants, regardless of whether cells were derived from control or theophylline-fed animals.

Concentration-response curves for adenosine inhibition of isoproterenol-stimulated $I_{Ca}$ are shown in Figure 8. The concentration of adenosine required for half-maximal inhibition ($IC_{50}$) of isoproterenol-stimulated $I_{Ca}$ by adenosine was sixfold greater ($p<0.01$) in control (28.3±1.4 μM) than in heart cells from theophylline-fed guinea pigs (4.6±0.6 μM). This finding suggests that the increased number of adenosine receptors in treated animals is accompanied by a significant increase in the sensitivity of the cells to adenosine.

**Effect of theophylline pretreatment on cAMP in cardiomyocytes.** The effect of R-PIA on isoproterenol-stimulated cAMP accumulation in cells derived from control and theophylline-fed animals is shown in Figure 9. Data were pooled from four experiments, each assayed in quintuplicate. Average basal and isoproterenol-stimulated cAMP levels were 28.6±3.6 and 64.8±8.8 pmol/mg protein, respectively. A maximally effective concentration of R-PIA (0.1 mM) inhibited isoproterenol-stimulated cAMP accumulation to a greater extent in cardiomyocytes derived from theophylline-fed animals (42.0±4.2%) than control myocytes (28.7±1.8%). These data are consistent with the electrophysiological findings.

**Theophylline pretreatment does not affect the response of cardiomyocytes to isoproterenol and acetylcholine.** The effects of acetylcholine on isoproterenol-stimulated $I_{Ca}$ in cells from control versus treated animals were also analyzed (Figure 10). In this series of experiments it was confirmed that isoproterenol increased $I_{Ca}$ by an equivalent magnitude in myocytes derived from the control and treated groups (97±6% [n=8] vs. 103±9% [n=5], respectively; p>0.05). Furthermore, as illus-
trated in the example of Figure 10, submaximal concentration of acetylcholine (0.3 μM) inhibited isoproterenol-stimulated I_Ca in myocytes from control and theophylline-fed animals by essentially the same extent (48±7% vs. 51±6%, respectively; p>0.05). This is consistent with the finding that theophylline treatment had no effect on muscarinic receptors as assessed by [3H]QNB binding (Table 1).

Discussion

In this study, we investigated the dynamic regulation of the A1 adenosine receptors in guinea pig ventricular myocytes after chronic treatment with the adenosine antagonist theophylline. The main findings of the present study are that 1) cardiac adenosine receptors can be up-regulated by chronic exposure to an adenosine antagonist theophylline and 2) up-regulation is accompanied by enhanced sensitivity of ventricular myocytes to the electrophysiological and biochemical actions of adenosine receptor agonists. Consistent with the above findings are the recent observations of Szot et al.7 in which repeated (14 days) administration of theophylline resulted in an increase in the number of [3H]CHA binding sites to A1 adenosine receptors in rat brain and in a significant increase in seizure thresholds for convulsant agents. Thus, as in the present study, a theophylline-induced up-regulation of A1 receptors in brain tissue was associated with a functional change, that is, reduced sensitivity to convulsants.7 In addition, our results are also analogous to the finding that β-adrenergic receptors of heart are up-regulated after treatment of rats with propranolol or reserpine and that such hearts have an enhanced sensitivity to isoproterenol.25,26 However, there is an important distinction to be made in comparing the effects of myocardial β-adrenergic and A1 adenosine receptor antagonists. The sympathetic nervous system operates continuously to modulate heart rate, atrioventricular conduction, and contractility. Thus, it is predictable that blockade of this system would result in an up-regulation of receptors by antagonizing agonist-induced receptor down-regulation and desensitization.27 In contrast, whereas it is well established that adenosine accumulates during periods of hypoxia and ischemia,5,28 it is not clear or proven that adenosine levels in the hearts of normoxic animals such as those used in the present study are sufficient to occupy receptors. In fact, Newby29 has referred to adenosine as a “retaliatory metabolite,” a terminology chosen to emphasize that the concentration of

![Graph showing kinetics of inward calcium current inactivation in cardiac myocytes derived from control and theophylline-fed guinea pigs. Adenosine (ADO) and isoproterenol (ISO) had no significant effects on the fast and slow inactivation time constants of inward calcium current, which were nearly identical in ventricular myocytes derived from control (panels A, B, and C) and theophylline-fed animals (panels A', B', and C'). The results are typical of 13 experiments.](http://circres.ahajournals.org/)

FIGURE 7. Graphs showing kinetics of inward calcium current inactivation in cardiac myocytes derived from control and theophylline-fed guinea pigs. Adenosine (ADO) and isoproterenol (ISO) had no significant effects on the fast and slow inactivation time constants of inward calcium current, which were nearly identical in ventricular myocytes derived from control (panels A, B, and C) and theophylline-fed animals (panels A', B', and C'). The results are typical of 13 experiments.
Figure 8. Dose-response curves for adenosine-mediated inhibition of the isoproterenol-enhanced inward calcium current (I_{\text{Ca}}). The dose-response curve for adenosine inhibition of isoproterenol-enhanced I_{\text{Ca}} at given concentration was normalized with reference to the increase in I_{\text{Ca}} caused by isoproterenol (20 nM) as described under "Materials and Methods." The symbols represent the mean values from nine different cells from control animals (●) and eight different cells from theophylline-fed animals (○).

Figure 9. Bar graph showing effect of R-phenylisopropyladenosine (R-PIA) on isoproterenol (ISO)-stimulated cyclic AMP accumulation in guinea pig cardiac myocytes. Myocytes were prepared from the hearts of guinea pigs fed no drug (open bars, control) or theophylline (filled bars) for 7 days. There was no difference in either basal or ISO-stimulated cyclic AMP between the groups. The basal and ISO-stimulated cyclic AMP levels for both groups combined were 28.6±3.6 and 64.8±8.8 pmol/mg protein, respectively (n=8). The experimental protocol and assay procedures are described under "Materials and Methods." Data were pooled from four experiments. *Significant increase in the magnitude of the R-PIA response, p<0.05.

Theophylline, in addition to inhibiting adenosine receptor activation, can trigger translocation of intracellular calcium and inhibit cyclic nucleotide phosphodiesterases. However, the methylxanthine is more potent as an adenosine receptor antagonist than as a phosphodiesterase inhibitor, and Rail has pointed out that therapeutic concentrations of theophylline in humans (<50 μM) selectively antagonize adenosine receptors. Plasma concentrations of theophylline achieved in this study were less than 30 μg/ml (<170 μM). At 100 μM, the nucleoside is increased during state of stress (e.g., hypoxia and ischemia) to protect target cells (cardiac myocytes) from excessive external stimulation and which suggests by implication a minor role for adenosine in the absence of stress. On the other hand, adenosine levels in normoxic hearts are influenced by positive inotropic agents that increase oxygen demand and vary during the cardiac contraction cycle. In this study, the threshold concentration of adenosine required to inhibit isoproterenol-stimulated I_{\text{Ca}} was about 0.3 μM (Figure 8). The results of the present study are consistent with the possibility that the endogenous level of adenosine is sufficiently high in normoxic guinea pig hearts to partially occupy and down-regulate cardiac adenosine receptors. In fact, Cerbai et al also reported that the threshold concentration of adenosine required for attenuation of I_{\text{Ca}} in guinea pig atrial myocytes was 0.3 μM. In a recent study, it was found that interstitial adenosine levels (measured in epicardial porous disc) in the in situ guinea pig heart were 0.85±0.12 μM. Provided that this reflects the actual interstitial adenosine concentration, then the concentration of adenosine in normoxic guinea pig myocardium is sufficient to partially activate A₁ adenosine receptors. Thus, theophylline may act to antagonize the actions of endogenous adenosine and, consequently, produce an up-regulation of adenosine receptor number. An alternate possibility is that occupancy of cardiac A₁ adenosine receptors by theophylline causes receptor up-regulation even in the absence of an agonist. Although the latter hypothesis cannot be ruled out, we have found that theophylline-treatment fails to up-regulate adenosine receptor number in the hearts of chicken embryos. The data could be interpreted to indicate that guinea pig and embryonic chick hearts differ in their basal production of adenosine and that theophylline does not modify receptors in the absence of an agonist. In embryonic chick hearts, down-regulation of A₁ adenosine receptors and homologous desensitization of the negative inotropic response to adenosine due to chronic exposure of the heart to R-PIA has been observed. This latter finding has been also confirmed in atrial cells cultured from 14-day-old chick embryos. Theophylline binds the plasma proteins (60% at therapeutic levels in humans), it is unlikely that the free plasma concentrations of the methylxanthine in this study exceeded 100 μM.
Theophylline will occupy over 90% of $A_1$ adenosine receptors in guinea pig heart membranes ($K_D$ = 7.1 \mu M).\(^9\) Half-maximal inhibition of cAMP phosphodiesterase in guinea pig hearts requires 2 mM theophylline\(^{36}\); hence, 100 \mu M theophylline will inhibit the enzyme by less than 5%. In addition, at a concentration of 100 \mu M, aminophylline (theophylline ethylenediamine) had no effect on the cAMP content of isolated perfused guinea pig hearts.\(^4\) Theophylline also has been reported to enhance norepinephrine release from isolated guinea pig heart but, again, only in concentrations exceeding 100 \mu M.\(^3\)\(^9\) Thus, it is likely that the actions of theophylline observed in the present study resulted mainly from blockade of adenosine receptors and, hence, antagonism of cardiac actions mediated by $A_1$ adenosine receptors.

There has been some disagreement about whether one or two affinity states of adenosine receptors exist when assayed in the absence of guanine nucleotides. Using rat brain membranes, some investigators could detect only a single agonist binding site in the absence of guanine nucleotide\(^\text{13,40,41}\) whereas others have detected two agonist binding sites\(^\text{42-44}\). The number of $A_1$ receptors that bind agonists with high affinity is maximized in membranes by including Mg\(^2+\) and omitting guanine nucleotide during radioligand binding assays.\(^\text{10,11}\) Recently, Leid et al\(^{49}\) showed that the number of agonist radioligand binding sites in porcine atrial membranes measured in the presence of Mg\(^2+\) and the absence of guanine nucleotides is approximately equivalent to the number of antagonist radioligand binding sites. These data suggest that $A_1$ receptors in heart membranes are primarily found in a high-affinity agonist binding conformation when assayed under the conditions used in the present study. On the other hand, Martens et al\(^{46}\) could detect only a single low affinity binding site for the agonist radioligand $[^{125}]\text{I}$$\text{ABA}$, hydroxyphenylisopropyladenosine in intact rat ventricular myocytes, presumably due to effects of endogenous guanine nucleotides. Ideally, $A_1$ receptor number should be measured with antagonist radioligands. Unfortunately, currently available antagonist radioligands bind poorly to guinea pig heart membranes or intact cells. Thus, we are not certain if the change in high affinity binding sites for the agonist $[^{125}]\text{I}$$\text{ABA}$ is due to an increase in receptor number or to a change in number of receptors coupled to G proteins, which bind agonists with high affinity. A question as to the number of agonist-binding sites has also been an issue in the interpretation of the effects of caffeine on brain adenosine receptors. Feeding caffeine to rats for 1–3 weeks has been reported to increase the number of adenosine receptors by 20–25% in two studies\(^\text{6,40}\) but to shift receptors from a low to a high affinity state in a third study.\(^\text{47}\) Although the effect of theophylline to increase $[^{125}]\text{I}$$\text{ABA}$ binding in crude heart membranes could reflect either an increase in the number of receptors or a conversion of receptors from an undetected low affinity state to a high affinity state, the number of high affinity binding sites can be taken as an index of the number of “functional” receptors, that is, those receptors coupled to G proteins and responsible for the attenuation of the effects of isoproterenol on $I_{Ca}$.

The present results confirm that adenosine and acetylcholine attenuate isoproterenol-induced 1) increase in $I_{Ca}$ (Fig. A) and 2) prolongation of the action potential\(^1\) in guinea pig ventricular myocytes. It also confirms that neither isoproterenol nor adenosine changes the kinetics of $I_{Ca}$.\(^2\) Thus, the antagonism of adenosine is due to a decrease in maximal calcium channel conductance.\(^2\) This electrophysiological effect of adenosine is most likely due to an $A_1$ receptor–mediated decrease in isoproterenol-induced accumulation of cellular cAMP. This explanation and the results of the present study are consistent with the findings that forskolin-stimulated
I\textsubscript{Ca} and cAMP levels are both antagonized by adenosine, R-PIA\textsubscript{t}, and acetylcyanine.\textsuperscript{36,49} Previous studies have indicated that chronic caffeine ingestion reduces the number of \beta-adrenergic receptors in rat forebrain as measured by the B\textsubscript{max} for \textsuperscript{3}H]dihydroalprenolol binding.\textsuperscript{49} In this study, no apparent change in the number and/or affinity of \beta-adrenergic receptors was found in the hearts of animals treated with theophylline (Table 1), nor could any difference in electrophysiological and cAMP responses to isoproterenol be demonstrated in cardiac myocytes prepared from the two groups of animals (Figures 2, 6, and 9). Thus, these data suggest that it is unlikely that chronic treatment of guinea pigs with theophylline modifies the binding characteristics of \beta-receptors and/or sensitivity of the heart cells to \beta-adrenergic agonist. Likewise, neither \textsuperscript{3}H]QNB nor \textsuperscript{3}H]NBMP\textsubscript{R} (which labels nucleoside transport proteins) binding sites (number and/or affinity) were significantly different between cardiac membranes derived from control and theophylline-fed guinea pigs (Table 1). In keeping with this finding, the ability of acetylcyanine to antagonize the isoproterenol-stimulated I\textsubscript{Ca} was not altered (Figure 10). Therefore, the effects (increase in A\textsubscript{1} adenosine receptor number and sensitivity of myocytes to the electrophysiological actions of the nucleoside) of chronic treatment of guinea pigs with theophylline are specific for adenosine.

It is notable that although cardiac myocytes derived from theophylline-fed animals had enhanced sensitivity to adenosine, as measured by inhibition of isoproterenol-stimulated I\textsubscript{Ca}, the maximal effect of adenosine was not changed. Thus, the heart, by regulating receptor expression, may be able to modulate its sensitivity to adenosine without losing the capacity to respond maximally to the nucleoside during periods of severe hypoxic or ischemic stress.

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