Adenosine-Sensitive Afterdepolarizations and Triggered Activity in Guinea Pig Ventricular Myocytes

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This study examines the cellular basis and specificity of the effects of adenosine on early afterdepolarizations (EADs), delayed afterdepolarizations (DADs), and triggered activity (TA) induced by various drugs with different mechanisms of action. Membrane potential and currents were measured in isolated guinea pig ventricular myocytes. Adenosine (10–100 μM) significantly (p<0.05) reduced the amplitude of DADs and suppressed TA induced by isoproterenol (10–50 nM) and forskolin (1 μM) but not those induced by dibutyryl cAMP (1 μM), ouabain (1–5 μM), and 7.2 mM [Ca²⁺]₉. Adenosine also abolished EADs and TA induced by isoproterenol. In contrast, adenosine failed to abolish EADs and TA induced by quinidine (3 μM) or those that occurred spontaneously (i.e., in the absence of drugs). Transient inward current (Iₜ) was induced on repolarization after 2-second-long single depolarizing voltage steps or after 12-second-long trains of 300-msec depolarizing pulses. Concomitant with the attenuation of DADs, adenosine suppressed Iₜ caused by isoproterenol and forskolin but not those induced by ouabain, dibutyryl cAMP, and elevated [Ca²⁺]₉. The amplitude of Iₜ was dependent on the magnitude of the activating voltage step, but the suppression of Iₜ by adenosine was not. The selective A₁-adenosine receptor antagonist N-0061 (9-methyladenine derivative) antagonized the effects of adenosine on afterdepolarizations, Iₜ, and TA. In myocytes from guinea pigs treated with pertussis toxin, adenosine failed to attenuate DADs and Iₜ or abolish TA induced by isoproterenol or forskolin. In parallel experiments, isoproterenol (10 nM) raised cellular cAMP from 5.7±0.2 to 8.1±0.1 pmol and the selective A₁ receptor agonist cyclopentyladenosine (5 μM) reduced it to 6.5±0.2 pmol (p<0.05). Thus, adenosine specifically attenuates afterdepolarizations and abolishes TA by suppressing Iₜ that are associated with stimulation of adenylyl cyclase via a pertussis toxin–sensitive A₁ receptor–mediated action. In conclusion, the response of TA to adenosine may identify a mechanism of afterdepolarizations related to stimulation of adenylyl cyclase. (Circulation Research 1992;70:743–753)

KEY WORDS • action potentials • voltage clamp • adenosine receptor • triggered rhythms • arrhythmias • catecholamines • calcium overload

There has been increasing recognition of the role played by triggered activity caused by either early afterdepolarizations (EADs) or delayed afterdepolarizations (DADs) in the genesis of clinical arrhythmias.¹,² EADs that arise at membrane potentials near the action potential plateau appear to be due to time- and voltage-dependent reactivation of the L-type calcium current.³,⁴ On the other hand, DADs have been attributed to an oscillatory membrane current occurring on repolarization, hereinafter referred to as transient inward current (Iₜ).⁵ This current appears to be either a nonspecific cation current or a current generated by electrogenic Na-Ca exchange, and it is attributed to intracellular calcium overload and spontaneous oscillatory release of calcium from the sarcoplasmic reticulum.⁶,⁷

We previously proposed that the nucleoside adenosine may identify a subset of ventricular tachyarrhythmias that are catecholamine dependent (i.e., cAMP mediated).⁸ This was based on clinical and basic observations.⁸–¹⁰ For instance, adenosine effectively terminates exercise- or isoproterenol-induced ventricular tachycardias in patients with no structural heart disease, but it is ineffective in terminating reentrant ventricular tachycardia.⁸ In ventricular myocytes adenosine attenuates the increase in peak calcium inward current (I₉) caused by isoproterenol and forskolin.¹⁰,¹¹ This effect is associated with a decrease in cellular cAMP,¹⁰ at least in part caused by inhibition of adenylyl cyclase,¹²–¹⁴ and is the basis for the anti–β-adrenergic action of adenosine. These findings have led us to suggest that attenuation of isoproterenol-induced DADs and of triggered activity by adenosine is due to a decrease in cellular calcium overload.⁹ However, none of the above studies demonstrated the linkage between attenuation by adenosine of isoproterenol-induced Iₜ and the decrease in amplitude of DADs and termination of triggered activity. Likewise, the characterization of the effects of adenosine on

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afterdepolarizations (EADs and DADs) and triggered activity resulting from drugs that alter the intracellular Ca\(^{2+}\) load of myocytes via different mechanism(s) has not been investigated.

Thus, in the present study, we sought to 1) examine the effects of adenosine on EADs, DADs, and resulting triggered activity induced by various drugs with different mechanisms of action; 2) determine whether adenosine depresses I\(_{\text{K,}}\); and 3) establish the role of an inhibitory guanine nucleotide binding protein (G protein) as mediator of the effects of adenosine by using pertussis toxin (PTX). Finally, because adenosine inhibition of adenylate cyclase can be mediated by activation of either the cell surface extracellular Ca\(^{2+}\)-adenosine receptor\(^{14}\) or the intracellular purine (P) site,\(^{15}\) the site of adenosine on afterdepolarizations and triggered activity was also investigated.

Materials and Methods

Chemicals

Adenosine, N\(^6\)-cyclopentyladenosine (CPA), 8-cyclopentyltheophylline (CPT), ouabain, forskolin, diodeoxyadenosine (DDA), and bovine serum albumin were purchased from Sigma Chemical Co., St. Louis, Mo. Dispase and N\(^6\),2'-O-dibutyryladenosine 3',5'-cyclic monophosphate (DBcAMP) were purchased from Boehringer Mannheim, Indianapolis, Ind. Collagenase was purchased from Worthington Biochemical Corp., Freehold, N.J., and trypsin from Serva Fine Biochemicals, Heidelberg, FRG. PTX was purchased from Calbiochem Corp., San Diego, Calif., and (+)-N\(^6\)-endo-norbornan-2-yl-9-methyladenine (N-0861) was a gift from Whitby Research Inc., Richmond, Va.

Solutions

Krebs-Henseleit (K-H) solution contained (mM) NaCl 127, KCl 4.6, CaCl\(_2\) 2, MgSO\(_4\) 1.1, sodium pyruvate 2, glucose 10, creatine 10, taurine 20, ribose 5, adenine 0.01, allopurinol 0.1, and HEPES 5, adjusted with NaOH to pH 7.4. The composition of the Ca\(^{2+}\)-free solution was the same as that of the K-H solution except for the deletion of CaCl\(_2\). The enzyme solution contained 0.4 mg/ml collagenase (type II), 0.04 mg/ml dispase, 0.04 mg/ml trypsin, and 2 mg/ml albumin, in Ca\(^{2+}\)-free solution. The conventional micropipettes were filled with 3 M KCl, whereas the “patch electrodes” were filled with a solution (i.e., internal pipette solution) containing (mM) KCl 10, potassium aspartate 130, Na\(_2\)ATP 4, MgCl\(_2\) 1, and HEPES 1, and pH was adjusted to 7.2 with KOH. Note that EGTA, which is a normal constituent of internal pipette solutions, had to be omitted because I\(_{\text{K,}}\) could not be reproducibly induced by isoproterenol or ouabain when EGTA was present.

Isolation Procedure

Single ventricular myocytes were prepared from adult Hartley guinea pigs of either sex (weighing 300–500 g). In a separate series of studies, single ventricular myocytes were isolated from guinea pigs injected with PTX (150 \(\mu\)g/kg) via the jugular vein 72 hours before the experiments. Briefly, the hearts from control (untreated) and PTX-treated guinea pigs were perfused with the following solutions gassed with 100% O\(_2\) at 35\(^\circ\)C and at a rate of 8 ml/min: 1) K-H solution for 10 minutes followed by 2) Ca\(^{2+}\)-free solution for 10 minutes and 3) enzyme solution for another 20 minutes. After perfusion with the enzyme solution, the ventricles were removed, minced, and gently shaken for 10–20 minutes. The cells were harvested from the suspension, filtered, and stored at room temperature in K-H solution containing 0.1 mM Ca\(^{2+}\).

Electrophysiological Techniques

Isolated ventricular myocytes were transferred into a recording chamber that was mounted on the stage of an inverted microscope coupled to a video camera system. Cells were continuously superfused with K-H solution at a rate of about 2 ml/min. The bath temperature in all experiments was maintained at 34.5±0.5\(^\circ\)C. Transmembrane action potentials and currents were measured with conventional single microelectrodes filled with 3 M KCl and resistances of 30–50 M\(\Omega\) by using an Axoclamp Model 2A (Axon Instruments, Burlingame, Calif.). To facilitate the development of DADs and I\(_{\text{K,}}\), 15-second-long drives at a rate of 2 Hz and 12-second-long trains of 300-msec depolarizing voltage-clamp steps from −80 to +40 mV were applied, respectively. The action potentials and current tracings were directly recorded on paper with a two-channel Gould (model 2200S) chart recorder. Action potential durations were measured at 50% (APD\(_{50}\)) and 90% (APD\(_{90}\)) of repolarization. The amplitude of DAD was taken as the difference between the maximum diastolic potential and the peak of the first DAD after the stimulation train. I\(_{\text{K,}}\) was measured from the holding current to the peak inward current after the last depolarizing clamp pulse.

In experiments designed to investigate the effects of adenosine on the I\(_{\text{K,}}\) induced by isoproterenol and ouabain, the whole-cell voltage-clamp method, as described by Hamill et al.,\(^{16}\) was used. The patch electrodes had resistances of 2–3 M\(\Omega\) when filled with the internal pipette solution described above. The junctional potential was corrected by zeroing the current before the microelectrode contacted the surface of the myocyte. When the cell membrane was ruptured, the holding potential was set at the zero current level (resting membrane potential). Recordings were obtained in voltage-clamp mode by using an Axoclamp Model 1C (Axon). To inactive the fast sodium current, the holding potential was set at −40 mV. To construct the current–voltage relations for I\(_{\text{K,}}\) 2-second-long depolarizing voltage-clamp pulses from holding potential to potentials ranging between −30 and +80 mV were applied at a rate of 0.1 Hz. Membrane currents were displayed on a storage oscilloscope (model 5113, Tektronix, Beaverton, Ore.) and also digitized at the sampling frequency of 40 kHz on-line using a 25-MHz Northgate Computer (model Slimline 386, Minneapolis, Minn.) and pCLAMP software version 5.5 from Axon Instruments, Foster City, Calif.

cAMP Assay

Guinea pig ventricular myocytes, isolated by the procedure described above, were allowed to settle at room temperature in K-H solution before drug incubation. With the supernatant removed, the cell suspension was concentrated to about 1.6×10\(^5\) rod-shaped cells per milliliter and
then aliquoted (400 µl). At a constant temperature of 35°C, the cells were pretreated with CPT and/or CPA or vehicle, as indicated, for 2 minutes and then incubated with isoproterenol for another 2 minutes. At the end of the incubation, 592 µl of 85 mM HCl was added to each sample to stop the reaction and lyse the cells. A 500-µl aliquot of each sample was acetylated and analyzed for cAMP content by using the automated radioimmunoassay method of Brooker et al.17

Data Analysis

Values are given as mean±SEM. The paired Student’s t test and Duncan’s multiple range test were used for the statistical analysis. Differences between values were considered statistically significant at p<0.05.

Results

General

Ventricular myocytes superfused with the normal K-H solution (4.6 mM K+ and 2.0 mM Ca2+) at 34.5±0.5°C had a resting membrane potential of −84±3 mV. On stimulation (0.25 Hz), transmembrane action potentials were elicited. The action potential amplitudes were 130±3 mV and had overshoots of +40±2 mV. APD50 and APD90 were 204±10 and 236±10 msec (n=20), respectively.

Afterdepolarizations and triggered activity, which were rarely observed during control conditions (i.e., absence of drugs) could be reproducibly induced by isoproterenol, forskolin, ouabain, DBcAMP, and quindine and by raising the extracellular calcium concentration from 2.0 to 7.2 mM. The afterdepolarizations and triggered activity caused by the various drugs sometimes appeared during stimulation of the myocytes at a rate of 0.25 Hz but were more easily elicited with overdrive stimulation (15-second-long drive at 2 Hz). Coincident with the appearance of DADs, in the same myocytes, ITi could be induced with 12-second-long trains of 300-msec depolarizing pulses from a holding potential of −80 to +40 mV (see “Materials and Methods”).

Afterdepolarizations and Triggered Activity Induced by Isoproterenol and Forskolin

Isoproterenol. In the presence of isoproterenol (10–50 nM), overdrive stimulation reproducibly elicited DADs (Figures 1 and 2, Table 1), which sometimes developed into triggered activity. Adenosine attenuated the isoproterenol-induced DADs and ITi (Figure 1, Table 1) and terminated triggered activity. Adenosine (10–100 µM) significantly reduced the amplitude of DADs and ITi by 87% and 72%, respectively (Table 1). In some myocytes, isoproterenol elicited DADs, EADs, and triggered activity without requiring overdrive stimulation (Figure 2). Adenosine also attenuated and abolished these afterdepolarizations and triggered activity (Figure 2C). Consistent with its anti-β-adrenergic effects on the afterdepolarizations, ITi and triggered activity, in the same myocytes, adenosine significantly reduced isoproterenol-induced prolongation of APD50 and APD90 by 50% and 56%, respectively (Figure 3A). As shown in Table 2 and Figures 1 and 2, the inhibitory effects of adenosine on isoproterenol-induced DADs and ITi were significantly antagonized by the A1-selective non-xanthine receptor blocker N-0861 (Figure 1D) and by the xanthine derivative CPT (Figure 2D). Likewise, inhibition by adenosine of the isoproterenol-induced prolongation of the APD50 and APD90 was antagonized 100% and 96%, respectively, by 5 µM CPT or 10 µM N-0861 (Figure 3A). Not shown, neither N-0861 nor CPT alone or in combination with isoproterenol caused any significant detectable change in action potential configuration. That is, these compounds only antagonized the effects of adenosine.

Forskolin. As in the case of isoproterenol, adenosine significantly diminished the amplitude of DADs and ITi caused by 1 µM forskolin by 98% and 93%, respectively (Table 1). In the same myocytes, adenosine shortened forskolin-induced prolongation of APD50 and APD90 by as much as 72% and 73%, respectively (Figure 3B). Similar to isoproterenol, the effects of adenosine on forskolin-induced DADs, ITi and pro-
longation of action potential duration were reversed by 5 μM CPT (Table 2, Figure 3B).

**Afterdepolarizations and Triggered Activity Induced by Ouabain, Elevated [Ca\textsuperscript{2+}]\textsubscript{o}, Dibutyryl cAMP, or Quinidine**

The amplitude of DADs and \( I_{Ti} \) in the presence of 1–5 μM ouabain increased as a function of exposure time to the cardiac glycoside until triggered activity ensued. Increasing the concentration of calcium in the superfusion medium to 7.2 mM or addition of DBcAMP (1 μM) also reproducibly induced DADs and \( I_{Ti} \). In addition, DBcAMP prolonged the action potential duration (APD\textsubscript{90} and APD\textsubscript{90}), whereas ouabain and elevated [Ca\textsuperscript{2+}]\textsubscript{o} shortened it (not shown).

In contrast to DADs, \( I_{Ti} \) and triggered activity induced by isoproterenol and forskolin, those induced by ouabain, elevated [Ca\textsuperscript{2+}]\textsubscript{o}, or DBcAMP were not inhibited by adenosine (Table 1). Adenosine at a concentra-

**TABLE 1. Effect of Adenosine on the Amplitude of Delayed Afterdepolarization and Transient Inward Current in Guinea Pig Ventricular Myocytes**

<table>
<thead>
<tr>
<th></th>
<th>DAD (mV)</th>
<th>( I_{Ti} ) (pA)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Adenosine</td>
</tr>
<tr>
<td>Isoproterenol</td>
<td>8.5±1.5</td>
<td>1.0±0.5*</td>
</tr>
<tr>
<td>(10–50 nM)</td>
<td>(12)</td>
<td>(12)</td>
</tr>
<tr>
<td>Forskolin</td>
<td>5.5±2.0</td>
<td>0*</td>
</tr>
<tr>
<td>(1 μM)</td>
<td>(7)</td>
<td>(7)</td>
</tr>
<tr>
<td>Ouabain</td>
<td>5.5±1.5</td>
<td>7.5±2.0</td>
</tr>
<tr>
<td>(1–5 μM)</td>
<td>(5)</td>
<td>(5)</td>
</tr>
<tr>
<td>DBcAMP</td>
<td>2.5±1.0</td>
<td>2.5±1.4</td>
</tr>
<tr>
<td>(1 μM)</td>
<td>(4)</td>
<td>(4)</td>
</tr>
<tr>
<td>[Ca\textsuperscript{2+}]\textsubscript{o}</td>
<td>3.0±0.5</td>
<td>3.5±0.5</td>
</tr>
<tr>
<td>(7.2 mM)</td>
<td>(5)</td>
<td>(5)</td>
</tr>
</tbody>
</table>

Values are mean±SEM. Numbers in parentheses are the numbers of cells studied. DAD, delayed afterdepolarization; \( I_{Ti} \), transient inward current; DBcAMP, dibutyryl cAMP. DADs were measured at the end of a 15-second-long drive at a rate of 2 Hz. \( I_{Ti} \) was measured at the end of a 12-second-long train of 300-msec depolarizing voltage-clamp steps from −80 to +40 mV. Measurements were made at steady-state effects of the drugs.

*Values significantly different from control \((p<0.05)\).
TABLE 2. Reversal of the Effects of Adenosine on the Amplitude of Delayed Afterdepolarization and Transient Inward Current by Adenosine Receptor Antagonists in Guinea Pig Myocytes

<table>
<thead>
<tr>
<th></th>
<th>DAD (mV)</th>
<th>I\textsubscript{T} (pA)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Isoproterenol</td>
<td>Forskolin</td>
</tr>
<tr>
<td>Control</td>
<td>8.4±2.0</td>
<td>3.0±0.7</td>
</tr>
<tr>
<td></td>
<td>(6)</td>
<td>(4)</td>
</tr>
<tr>
<td>Adenosine</td>
<td>1.0±0.7*</td>
<td>0.2±0.2*</td>
</tr>
<tr>
<td></td>
<td>(6)</td>
<td>(4)</td>
</tr>
<tr>
<td>+CPT</td>
<td>6.5±1.7†</td>
<td>5.8±1.7†</td>
</tr>
<tr>
<td>Adenosine</td>
<td>7.3±1.7†</td>
<td>...</td>
</tr>
<tr>
<td>+N-0861</td>
<td>(6)</td>
<td>(5)</td>
</tr>
</tbody>
</table>

Values are mean±SEM. Numbers in parentheses are the numbers of cells studied. DAD, delayed afterdepolarization; I\textsubscript{T}, transient inward current; CPT, cyclopentyltheophylline; N-0861, N\textsuperscript{6}-endo-dornoryl-9-methyladenine. DADs were measured at the end of a 15-second-long drive at a rate of 2 Hz. I\textsubscript{T} was measured at the end of a 12-second-long train of 300-msec depolarizing voltage-clamp steps from −80 to +40 mV. Measurements were made at steady-state effects of the drugs. The concentrations of the drugs were as follows: isoproterenol, 10 nM; forskolin, 1 μM; adenosine, 100 μM; CPT, 5 μM; and N-0861, 10 μM.

A. CONTROL

B. QUINIDINE

C. QUINIDINE + ADO

D. QUINIDINE + 5mM[Mg\textsubscript{J}]\textsubscript{0}

**Figure 4.** Lack of effect of adenosine (ADO) on early afterdepolarizations caused by quinidine. Action potentials were recorded from cells stimulated at a basal rate of 0.25 Hz throughout the experiment. Panels A–C: Action potentials are from a single ventricular myocyte. Arrows point to early afterdepolarizations. Quinidine-induced early afterdepolarizations were not attenuated by ADO (panel C) but were abolished by raising the concentration of magnesium (panel D). Calibration marks in panel D apply to all panels.

The mechanism underlying the functional antagonism between adenosine and catecholamines is at least in part due to inhibition of the cAMP-producing enzyme adenylate cyclase. 12–14 This inhibitory effect of adenosine is presumably mediated by the extracellular A\textsubscript{1} adenosine receptor but could also involve the inhibitory intracellular P site. 35 Thus, studies were performed to determine the effects of the adenosine P site agonist DDA on isoproterenol-induced afterdepolarizations, triggered activity, and I\textsubscript{T}. In addition, to determine absence and presence of adenosine was determined. Figure 5 shows membrane currents elicited by a 2-second-long depolarizing clamp pulse to +20 mV from a holding potential of −40 mV. During control conditions (i.e., absence of drugs), I\textsubscript{T} was not observed (Figure 5, top tracing). In the presence of 10 nM isoproterenol (within 2–3 minutes of application), the amplitude of I\textsubscript{T} increased and I\textsubscript{T} was observed after the depolarizing clamp step (Figure 5, middle tracing). The addition of 100 μM adenosine, still in the presence of isoproterenol, rapidly abolished I\textsubscript{T} and reduced the amplitude of I\textsubscript{T} to baseline (Figure 5, bottom tracing). The relation between the voltage of the depolarizing step and the magnitude of I\textsubscript{T} induced by isoproterenol (Figure 6A) and ouabain (Figure 6B) is sigmoidal, with an activation threshold between −20 and −10 mV, and maximal current was observed at voltages greater than +40 mV. Adenosine (100 μM), independent of membrane potential, abolished I\textsubscript{T} induced by isoproterenol (Figure 6A), whereas the amplitude of ouabain-induced I\textsubscript{T} was unaffected by adenosine (Figure 6B).

**Mechanism of Action of Adenosine on Delayed Afterdepolarizations, Triggered Activity, and Transient Inward Current Caused by Isoproterenol**

The mechanism underlying the functional antagonism between adenosine and catecholamines is at least in part due to inhibition of the cAMP-producing enzyme adenylate cyclase. This inhibitory effect of adenosine is presumably mediated by the extracellular A\textsubscript{1} adenosine receptor but could also involve the inhibitory intracellular P site. Thus, studies were performed to determine the effects of the adenosine P site agonist DDA on isoproterenol-induced afterdepolarizations, triggered activity, and I\textsubscript{T}. In addition, to determine

**Dependence of Transient Inward Current Induced by Isoproterenol and Ouabain on the Amplitude of the Voltage-Clamp Step**

To further elucidate the underlying electrophysiologic basis of adenosine’s effect on DADs, the influence of the depolarizing clamp step voltage on the amplitude of I\textsubscript{T} induced by isoproterenol and ouabain in the...
whether the electrophysiological antagonism of catecholamines by adenosine is associated with changes in cellular cAMP, the effects of the A1-adenosine selective agonist CPA on isoproterenol-induced cAMP accumulation in ventricular myocytes were investigated. Finally, to establish the involvement of an inhibitory G protein mediating the effects of adenosine, experiments were carried out in myocytes isolated from guinea pigs pretreated with PTX.

**Role of the intracellular P site.** Stimulation of the intracellular P site is another mechanism whereby adenosine can modulate adenylate cyclase. In a separate series of experiments, the effects of the P site selective agonist DDA, applied extracellularly and intracellularly, on isoproterenol-induced afterdepolarizations and \( I_{\text{f}} \) were investigated. As summarized in Figure 7, DADs and \( I_{\text{f}} \) (not shown) caused by 10 nM isoproterenol were not attenuated by 100 \( \mu \)M DDA applied extracellularly but were abolished by 100 \( \mu \)M adenosine. The potential role of the intracellular P site in mediating the actions of adenosine was further examined by intracellular application of DDA (Figure 8). In these experiments (\( n=3 \)), the recording microelectrodes (patch electrodes) were filled with a solution containing 100 \( \mu \)M DDA. In the example illustrated in Figure 8, triggered activity induced in the presence of isoproterenol was not slowed or terminated by internal perfusion of the myocyte with DDA, but it was abolished by extracellular application of adenosine (10 \( \mu \)M).

**Changes in cellular cAMP.** The effect of the A1-adenosine agonist CPA and of the adenosine receptor antagonist CPT on isoproterenol-stimulated cAMP accumulation in ventricular myocytes was also investigated. The control (basal) cAMP level was 5.69±0.18 pmol. Isoproterenol (10 nM) alone caused a significant increase in cellular cAMP to 8.14±0.11 pmol. The addition of 5 \( \mu \)M CPA, in the continued presence of isoproterenol, significantly reduced the cellular cAMP accumulation to 6.55±0.19 pmol. CPA alone had no significant effect on the basal levels of cAMP. The attenuation by CPA of isoproterenol-induced increase in cellular cAMP accumulation was completely antagonized by the adenosine antagonist CPT. That is, in the presence of isoproterenol plus CPA, the addition of 10 \( \mu \)M CPT caused a significant increase in cAMP, from 6.55±0.19 to 8.90±0.20 pmol. These data on cAMP levels are consistent with the effects of adenosine on...
isoproterenol-induced afterdepolarizations, triggered activity, and $I_{h}$ and with the reversal of depressant effects of adenosine by the adenosine receptor antagonists N-0861 (Figure 1, Table 2) and CPT (Figure 2, Table 2).

Effect of pertussis toxin pretreatment. Adenosine-mediated opening of $K^{+}$ channels in atrial myocytes and inhibition of isoproterenol-stimulated $I_{h}$ in ventricular myocytes depend on an inhibitory PTX-sensitive guanine nucleotide binding protein $G_{i}$. Therefore, to establish that adenosine’s effects on afterdepolarizations, triggered activity, and $I_{h}$ elicited in the presence of isoproterenol are also mediated by $G_{i}$, we investigated the effects of PTX on adenosine. In ventricular myocytes isolated from guinea pigs pretreated with PTX, adenosine (100 µM) did not decrease the amplitude of DADs and $I_{h}$ caused by 10 nM isoproterenol (Figure 9) or abolish triggered activity (not shown). As illustrated in Figure 9, although the amplitudes of the DADs and $I_{h}$ induced in the presence of isoproterenol were similar in myocytes from control and PTX-treated animals, adenosine reduced only those DADs and $I_{h}$ induced in myocytes isolated from control guinea pigs.

**Discussion**

The major new finding of the present study is that adenosine specifically attenuates and abolishes DADs and EADs and triggered activity induced by agents (e.g., isoproterenol) known to stimulate the enzyme adenylate cyclase and thereby raise cellular cAMP. The underlying electrophysiological basis for this effect of adenosine is the suppression of $I_{h}$, an effect that presumably is due to a decrease in cellular calcium load. The results of the present study also demonstrate that the effect of adenosine on afterdepolarizations, triggered activity, and $I_{h}$ is mediated by the inhibitory extracellular $A_{1}$ adenosine receptor and not the intracellular P site and is dependent on a PTX-sensitive guanine nucleotide binding protein.

**Specificity of Adenosine Action**

As depicted in Figure 10, $I_{h}$, DADs, and triggered activity can be elicited by various drugs and interventions that increase [Ca$^{2+}$], and result in oscillatory release of Ca$^{2+}$ from sarcoplasmic reticulum by one of several mechanisms. Adenosine appears to specifically modulate the effects of only those drugs that stimulate the enzyme adenylate cyclase (Figure 10). This conclusion is based on the lack of effect of adenosine on $I_{h}$ and afterdepolarizations elicited by drugs and interventions that do not stimulate adenylate cyclase and raise cellular cAMP. These results and interpretations are in keeping with at least two previous observations. That is, in ventricular myocytes 1) adenosine produces a negative inotropic effect only under conditions in which the adenylate cyclase-cAMP system has been stimulated, such as in the presence of isoproterenol and forskolin, and 2) adenosine reduces isoproterenol- and forskolin-stimulated $I_{Ca}$ but does not attenuate basal $I_{Ca}$. Adenosine did not attenuate afterdepolariza-
Figure 9. Bar graph showing lack of effect of adenosine (ADO) on isoproterenol (ISO)-induced delayed afterdepolarization (DAD) and transient inward current (I_{Ti}) in ventricular myocytes from pertussis toxin (PTX)-treated guinea pigs. Summary of the data on the effect of ADO on ISO-induced DADS and I_{Ti} in myocytes from control (n=12) and PTX-treated (n=7) guinea pigs. Each bar represents the mean±SEM. *Values significantly different (p<0.05) from ISO alone. NS, differences between ISO+ADO vs. ISO alone were not significant.

Figure 10. Schematic diagram of the mechanisms for the induction of transient inward current (I_{Ti}), delayed afterdepolarization (DAD), triggered activity (TA), and aftercontraction in cardiomyocytes. Adenosine via A_{1} receptor coupled to the inhibitory G protein (G_{i}) inhibits adenylyl cyclase (AC) activity, lowers cellular cAMP, and thereby attenuates and abolishes I_{Ti}, DADs, aftercontractions, and TA induced by isoproterenol and forskolin. See text for details. DBcAMP, dibutyryl cAMP; S.R., sarcoplasmic reticulum.

In this study, adenosine does not attenuate EADs or DADs or terminate triggered activity occurring under basal conditions, that is, in the absence of any drugs. As to the mechanism by which quinidine induces EADs that arise at plateau voltages, it has been proposed that by retarding action potential repolarization quinidine increases the degree of recovery from inactivation of L-type Ca^{2+} channels, which allows their reactivation and, in turn, development of EADs. In addition, the marked prolongation of the action potential duration caused by quinidine should also contribute to intracellular Ca^{2+} overload. Regardless of the underlying electrophysiological basis for the EADs induced by quinidine, the mechanism(s) of action of this drug does not involve stimulation of adenylyl cyclase, which explains the lack of effect of adenosine. In contrast, EADs elicited by isoproterenol, which are associated with stimulation of adenylyl cyclase and subsequent increase in cellular cAMP, are attenuated or abolished by adenosine (Figure 4). Taken together, the above results lead to the conclusion that adenosine, because of its highly specific mode of action, provides for a mechanistic differentiation of the underlying biochemical basis of afterdepolarizations and triggered activity. The response of DADs, EADs, or triggered activity to adenosine provides means to distinguish afterdepolarizations that are dependent on stimulation of adenylyl cyclase from those that are not. This conclusion is applicable only to ventricular and not atrial myocytes in which adenosine can directly (i.e., independent of changes in cellular cAMP) activate the potassium outward current (I_{Karb}).

Suppression of Transient Inward Current

DADs induced by elevated [Ca^{2+}]_{o}, and the various drugs used in the present study were associated with I_{Ti}. Likewise, the effect of adenosine or the lack of it on DADs paralleled that on I_{Ti}. Although these observations do not prove a cause and effect relation, they strongly support the hypothesis that suppression of I_{Ti} is the basis for the inhibitory effects of adenosine on afterdepolarizations and triggered activity. Additional evidence in support of this hypothesis is derived from the experiments in which DADs and I_{Ti} elicited in the same myocytes, were recorded at the end of a 15-
second-long train of action potentials (overdrive stimulation) and depolarizing voltage-clamp steps, respectively. Because DADs and I_{Na} were recorded under similar experimental conditions and in the same myocytes, a more direct comparison between them should be possible. Moreover, the depolarizing voltage-clamp steps from −80 to +40 mV and 300 msec in duration are in the same range as the resting potential, overshoot, and action potential duration (APD_{90}) recorded during isoproterenol, forskolin, and DBcAMP. The relation of I_{Na} to adenosine's inhibition of isoproterenol-induced DADs is also supported by the results of the experiments that investigated the influence of the activating depolarizing step on the amplitude of I_{Na} (Figure 6). The effect of adenosine on I_{Na} induced in the presence of isoproterenol (Figure 6A) or lack of it in the case of ouabain (Figure 6B) was independent of the activating voltage-clamp step. The dependence of the magnitude of isoproterenol- and ouabain-induced I_{Na} on the amplitude of the depolarizing clamp step is similar to results described for I_{Na} induced bystrophanthin and more recently rose bengal in ventricular myocytes from guinea pig and rabbit hearts, respectively.

Detection and quantification of I_{Na} may be affected by activation of other ion currents. However, the closely parallel responses of DADs and I_{Na} to adenosine are too coincidental to simply be attributed to changes in some contaminating current(s). Therefore, suppression of I_{Na} is the most plausible explanation for the attenuation and abolition of DADs and triggered activity by adenosine.

Cellular calcium overload followed by spontaneous oscillatory release of calcium from sarcoplasmic reticulum appears to induce a nonspecific cation current and/or a Na-Ca exchange current referred to as I_{Ca}. In this context, adenosine, by attenuating the increase in I_{Ca} and the prolongation of action potential caused by isoproterenol and forskolin, is expected to renormalize the excess cellular calcium load produced by these agents. Hence, suppression of I_{Na} by adenosine may reflect a reduction in cellular calcium overload and thereby a decrease in the spontaneous oscillatory release of calcium from the sarcoplasmic reticulum. In the present study, increased cellular calcium influx to raise [Ca^{2+}]_{i} may have occurred via different mechanisms depending on the drug used to elicit I_{Na} (Figure 10). In the case of isoproterenol, as shown in Figure 5, enhanced cellular calcium influx occurred via an increase in I_{Ca} and prolongation of the action potential. Similarly, forskolin and DBcAMP have also been shown to increase I_{Ca} and prolong the action potential duration in ventricular myocytes. Ouabain, increased cellular calcium influx, and/or decreased cellular efflux most likely occurred via an increase in Na-Ca exchange caused by a rise in intracellular sodium. Thus, the stimulation of I_{Ca}, Na-Ca exchange, and the prolongation of the action potential may alone, or combined, create a state of cellular calcium overload, which in turn leads to activation of I_{Na}. In the present study, cellular overload was presumably further exacerbated by the 15-second-long overdrive stimulation or depolarizing voltage-clamp steps. As for quinidine, as discussed above, prolongation of the action potential repolarization should accelerate L-type I_{Ca} recovery and hence lead to increased cellular calcium influx.

The evidence for increased cellular calcium load with the drugs used in the present study is based on the observation (not shown) that DADs were associated with aftercontractions (detected with a cell-edge tracking device). In addition, we previously showed that DADs and aftercontractions induced with isoproterenol are both attenuated by adenosine (Figure 8 of Reference 9). However, this is only indirect evidence and further experiments in which intracellular calcium is measured directly will be required to conclusively demonstrate that adenosine reduces cellular calcium load.

**Mechanism of Adenosine Action**

Modulation of adenylate cyclase activity and cellular cAMP levels by adenosine is mediated by extracellular adenosine receptors (A_{1} and A_{2} subtypes) and via the intracellular P site. Activation of the A_{2}-adenosine receptor and the P site inhibits, whereas activation of the A_{2}-adenosine receptor stimulates, adenylate cyclase activity with corresponding changes in cellular cAMP.

A_{2}-adenosine receptor. In contrast to activation of the potassium outward current, I_{Kadou} which is independent of cAMP, attenuation by adenosine of isoproterenol- and forskolin-stimulated I_{Ca} is due to inhibition of adenylate cyclase and is accompanied by a concomitant reduction in cellular cAMP levels. In the present study, these previous observations were not only confirmed but also extended to include adenosine's effect on I_{Na} and resulting DADs. Furthermore, the results lead to the conclusion that inhibition by adenosine of I_{Na} and DADs elicited in the presence of isoproterenol and forskolin is mediated by the cell surface A_{1} receptor subtype, which is coupled to an inhibitory PTX-sensitive guanine nucleotide binding protein, most likely G_{i}. Evidence implicating the extracellular A_{1}-adenosine receptor as the mediator of the effects of adenosine in this study is the complete reversal of adenosine's effect by the antagonists CPT and N-0861. The xanthine CPT is a potent (pA_{2} value of 7.5) and competitive adenosine antagonist that is slightly selective for A_{1} receptors (i.e., with an affinity A_{1}/A_{2} of 140). In comparison, in the heart, the newly developed nonxanthine adenosine antagonist N-0861, which is also a competitive antagonist, is less potent (pA_{2} value of 6.2) than CPT but is devoid of functional A_{2} receptor antagonist activity. That is, pharmacologically, N-0861 behaves as a pure A_{1}-adenosine antagonist, which does not attenuate the coronary vasodilation (A_{2} receptor-mediated effect) caused by adenosine.

The failure of adenosine to attenuate and abolish isoproterenol- and forskolin-induced I_{Na} DADs, and triggered activity in myocytes obtained from PTX-treated guinea pigs indicates that adenosine's effects are mediated by a receptor coupled to the inhibitory G_{i} protein. Consistent with an A_{2}-adenosine receptor-effector coupling mechanism that includes G_{i}, the selective A_{2}-adenosine receptor agonist CPA significantly reduced accumulation of cellular cAMP caused by isoproterenol. This effect of CPA on cAMP, like the electrophysiological effects of adenosine, was completely reversed by the adenosine antagonist CPT.

Thus, similar to other previously described cardiac electrophysiological effects of adenosine, the effect of adenosine on I_{Na} afterdepolarizations, and triggered
activity induced by isoproterenol and forskolin is mediated by $A_1$-adenosine receptors coupled to $G$. However, unlike the activation of the potassium outward current, $I_{K_Ado}$ in supraventricular tissues, which is also mediated by $A_1$-adenosine receptors coupled to $G$, but is independent of inhibition of adenylyl cyclase, the effect of adenosine on $I_{K_Ado}$, afterdepolarizations, and triggered activity in ventricular myocytes is dependent on inhibition of accumulation of the second messenger cAMP.

**Role of the intracellular P site.** Like the extracellular $A_1$-adenosine receptor, the intracellular P site, which is a distinct binding domain located in the catalytic subunit of adenylyl cyclase, inhibits the stimulated form of the enzyme and lowers cAMP levels. In isolated myocardial membranes prepared from rat hearts, LaMonica et al.13 found that $10^{-8} M$ of the P site agonist DDA caused a 19% inhibition of adenylyl cyclase activity previously stimulated with $10^{-6} M$ isoproterenol. The observation that this effect of DDA on adenylyl cyclase was not antagonized by either isobutylmethylxanthine or 8-phenyltheophylline was interpreted to indicate that the P site exists in myocardial membranes. However, the importance of this P site in modulation of functions dependent on adenylyl cyclase activity in intact cardiac cells has not yet been adequately established. That is, in heart muscle, the physiological role of the P site remains to be demonstrated. In the present study, DDA, a P site agonist, added to either the superfusion medium or the internal recording pipette solution (i.e., intracellularly) failed to inhibit isoproterenol-induced DADs, $I_{K_Ado}$, and triggered activity (Figures 7 and 8). Similarly, DDA did not attenuate the prolongation of the ventricular action potential caused by isoproterenol. Furthermore, the finding that both adenosine antagonists, that is, the $A_1$-adenosine selective N-0861 and nonselective CPT, completely reversed the effects of adenosine indicates that the observed actions of adenosine were entirely mediated by the extracellular $A_1$-adenosine receptor and not the intracellular P site.

**Clinical Implications**

The results of the present study clearly establish that in ventricular myocytes adenosine is highly specific for DADs, EADs, and resulting triggered activity caused by agents known to stimulate adenylyl cyclase and increase cellular cAMP. These results are consistent with clinical findings that show that adenosine is effective in terminating only exercise-induced ventricular tachycardia in patients without structural heart disease. Adenosine has no effect on ventricular tachycardia caused by reentry (associated with previous myocardial infarction) whether or not catecholamines facilitated. In conclusion, the response of triggered activity to adenosine may identify a mechanism of afterdepolarizations related to stimulation of adenylyl cyclase.

**Note added in proof.** While this manuscript was in press, the recent report of Fenton et al.14 came to our attention. These investigators presented evidence in support of the idea that in rat ventricular myocytes the anti-$\beta$-adrenergic action of adenosine is in part due to attenuation of isoproterenol-induced changes in intracellular $[Ca^{2+}]$. Furthermore, isoproterenol-induced spontaneous high frequency $Ca^{2+}$ transients were observed after antagonism of R-PIA, an $A_1$-adenosine receptor agonist, with a receptor antagonist. These results are consistent with our hypothesis that the suppression of isoproterenol- and forskolin-induced $I_{K_Ado}$ and triggered activity by adenosine is due to a reduction in cellular calcium and a decrease in the spontaneous oscillatory release of calcium from the sarcoplastic reticulum.

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**References**

5. Lederer WJ, Tsien RW: Transient inward current underlying arrhythmogenic effects of cardiotonic steroids in Purkinje fibers. J Physiol (Lond) 1979;263:73-100
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