Adenosine Enhances Neuroexcitability by Inhibiting a Slow Postspike Afterhyperpolarization in Rabbit Vagal Afferent Neurons

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Background—Electrophysiological mechanisms by which adenosine may activate cardiac afferent neurons are unknown. Slow afterhyperpolarizations (AHPs) follow action potentials in a subset of vagal C afferents, rendering them inexcitable. The purpose of this study was to test the hypothesis that adenosine increases vagal neuronal excitability by blocking slow AHPs and to determine the adenosine receptor subtype mediating these effects.

Methods and Results—Using the perforated patch-clamp technique, we identified cultured adult rabbit nodose ganglion cells with slow AHPs in current-clamp mode. Trains of 100 current pulses at 20% above threshold were injected, with an interspike interval of 100 ms, and the number of action potentials triggered were counted and reported as the action potential response rate. During adenosine (10 μmol/L), slow AHPs were suppressed and action potential response rate was augmented from 3.8±0.5% at baseline to 28±7% after adenosine (P=0.0009). The selective A2-adenosine receptor agonist NECA but not the A1-adenosine agonist CCPA replicated the adenosine effect. The selective A2A-adenosine antagonist ZM 241385 (10 nmol/L) but not the A1 adenosine antagonist DPCPX (5 μmol/L) abolished the adenosine effect. We considered two alternative hypotheses: (1) A2-receptor–mediated suppression of ICa leading to smaller increases in intracellular Ca during stimulation, resulting in less activation of IK(Ca) and consequent suppression of slow AHPs, or (2) A2-receptor–mediated elevation of cAMP directly suppressing slow AHPs. Under voltage-clamp conditions, adenosine did not significantly inhibit ICa, making the latter hypothesis more likely.

Conclusions—Adenosine inhibits slow AHPs in vagal afferent neurons. This effect is most likely caused by A2A-receptor–mediated stimulation of cAMP production. (Circulation. 2001;103:1325-1329.)

Key Words: adenosine ■ nervous system ■ receptors

adenosine is an endogenous nucleoside generated from the breakdown of ATP during myocardial ischemia. Most but not all experimental evidence in animals and humans suggests that adenosine released in the ischemic myocardium acts on cardiac afferent neurons to initiate systemic reflexes and to mediate cardiac pain. Thames and colleagues reported that myocardial ischemia in dogs reflexly increased renal sympathetic nerve activity. Sympathetic excitation was blunted in the presence of the adenosine antagonist aminophylline and augmented by dipyridamole, an inhibitor of adenosine uptake and elimination. In further experiments, an A1-receptor–selective agonist but not an A2-receptor–selective agonist reproduced the reflex sympathetic excitation. Transection of cardiac sympathetic afferent neurons eliminated this reflex increase in renal sympathetic nerve activity. These findings are consistent with activation of A1-adenosine receptors on sympathetic afferent neurons in the heart as mediators of reflex sympathetic excitation. In humans, intracoronary adenosine reproduces angina-like chest pain in the absence of ischemia. Intracoronary adenosine induces a reflex increase in blood pressure and plasma catecholamines. Chest pain during adenosine infusion or during exercise testing is decreased by infusion of aminophylline. On the other hand, an adenosine analogue administered intra-arterially to anesthetized cats had little if any effect on afferent nerve activity. In patch-clamping experiments, adenosine administered to rat nodose ganglion cells (cell body of vagal afferent neurons) does not elicit spontaneous action potentials.

The cellular electrophysiological mechanisms by which adenosine may activate cardiac afferent neurons to initiate systemic reflexes and elicit cardiac pain are unknown. Slow afterhyperpolarizations (AHPs) follow action potentials in a subset of vagal C afferent neurons, rendering them inexcitable. Attenuation of these slow AHPs increases neuronal excitability. Slow AHPs are mediated by IK(Ca) and are suppressed by cAMP. We have previously reported that adenosine, working on A1-adenosine receptors, attenuates the voltage-dependent IK(Ca) on a subset of cardiac vagal afferent neurons in rats. Therefore, we postulated that adenosine
A<sub>1</sub>-receptor activation may be neuroexcitatory by suppressing increases in intracellular calcium, thereby decreasing slow AHP amplitude. Alternatively, activation of A<sub>2</sub> receptors increases cAMP, which directly inhibits slow AHPs. The purpose of this study was to test the hypothesis that adenosine increases vagal neuronal excitability by blocking slow AHPs, to identify the adenosine receptor subtype mediating these effects, and to determine whether the underlying mechanism is mediated by suppression of calcium currents or cAMP.

**Methods**

**Tissue Culture**

Nodose ganglia from adult New Zealand White rabbits were isolated. Tissue culture was performed according to the method of Yoshimura et al. Cells were used for electrophysiological studies within 24 hours after isolation.

**Cellular Electrophysiology**

Perforated patch-voltage and current-clamp recordings were performed at room temperature. Patch electrodes had a resistance 2 to 3 MΩ. Membrane current and voltage were measured with an Axopatch 200 (Axon Instruments) patch-clamp amplifier controlled by a personal computer with a Digidata acquisition board driven by PCLAMP software (Axon Instruments). The standard external solution contained (in mmol/L) NaCl 136, KCl 5.4, NaH<sub>2</sub>PO<sub>4</sub> 0.33, MgCl<sub>2</sub> 1.0, HEPES 10.0, glucose 10.0, and CaCl<sub>2</sub> 1.8, pH 7.4. The internal solution contained (in mmol/L) KCl 140, CaCl<sub>2</sub> 1.0, MgCl<sub>2</sub> 0.5, HEPES 10.0, ATP 5, GTP 0.1, and cAMP 0.01. For isolation of calcium currents, Na and K in the external solution were replaced isotonically with Cs, 0.03 mmol/L tetrodotoxin (TTX) was added.

**Figure 1.** Adenosine (10 μmol/L) reversibly increases action potential response rate. A, Cell was stimulated at 20% threshold at 100-ms interspike intervals. At baseline, first 6 stimuli elicited action potentials, followed by hyperpolarization of cell membrane and failure to capture. B, In same cell, about 1 minute into adenosine (10 μmol/L) administration, 11 action potentials are elicited at beginning of train. Slow AHP is smaller and 6 more stimuli are followed by action potentials. C, Approximately 3 minutes into adenosine administration, slow AHP has been eliminated and there is 100% response rate to stimulus train. D, Approximately 2 minutes after washout of adenosine in same cell, first 8 stimuli elicited action potentials, followed by slow AHP and failure to capture. Dashed lines in each panel (labeled AP) show V<sub>max</sub> of action potentials. E, Baseline action potential response rate was 3.8±0.5%, which, after adenosine, increased to 28±7% (P<0.0009) and during washout returned to baseline levels (5±1%, P=NS). Note degree of augmentation was variable. Lines connect responses of individual neurons, open circles indicate mean±SD.

**Figure 2.** Forskolin (10 μmol/L) and cadmium (0.01 mmol/L) reversibly increase action potential response rate. Left, Baseline action potential response rate was 5±1.2%, which, after forskolin, increased to 41±17% (P<0.05) and then during washout returned to baseline levels (8.5±5%, P=NS). Right, Baseline action potential response rate was 4.4±0.9%, which, after cadmium, increased to 68±17% (P<0.01) and during washout returned to baseline levels (4.6±1%, P=NS). Patch was lost in some cells before washout data could be obtained.
and CaCl₂ was increased to 5 mmol/L; in the internal solution, K was replaced with 30 mmol/L tetraethylammonium (TEA) and 110 mmol/L Cs. Amphotericin (240 mg/mL) was used to perforate the cell membrane, as described previously. In current-clamp mode, action potentials were stimulated by current injection, and cells with slow AHPs were identified. Trains of 100 current pulses were injected at 20% above threshold, with an interspike interval of 100 ms. The number of action potentials elicited by the train was counted as a measure of excitability and reported as the action potential response rate. In voltage-clamp experiments, calcium currents were elicited by depolarizing voltage steps from a holding potential of −80 mV after blocking TTX-sensitive Na currents and K currents. Solution changes were performed with the aid of a rapid extracellular solution-exchange device. This system permits >90% exchange of the bath surrounding the neuron in <1 second.

Statistical Analysis
Data were analyzed by paired t tests and ANOVA.

Results

Effects of Adenosine on Action Potentials
The purpose of these experiments was (1) to determine if adenosine inhibited slow AHPs and increased vagal afferent excitability and (2) to determine if these slow AHPs were sensitive to forskolin (which augments cAMP) and/or cadmium (nonspecific IC₅₀ inhibitor) (Figure 1). A total of 18 cells with slow AHPs and 10 cells without slow AHPs were studied. During adenosine (10 μmol/L) administration, slow AHPs were suppressed, and the action potential response rate was augmented from 3.8±0.5% at baseline to 28±7% after adenosine (P=0.0009). Inhibition of slow AHPs and increased action potential response rate took several minutes to develop and largely reversed within 3 minutes of discontinuing adenosine (Figure 1). Although the action potential response rate was augmented by ≈10% in 17 of 18 cells, the degree of augmentation was variable (Figure 1), as has generally been observed in electrophysiological responses in this type of preparation. Adenosine had no effect on action potential response rate in cells without slow AHPs (data not shown).

Slow AHPs were reversibly inhibited by forskolin (10 μmol/L), resulting in an increased action potential response rate from 5±1.2% at baseline to 41±17% after forskolin, (n=5, P=0.05) (Figure 2A). Slow AHPs were also suppressed when cadmium (Cd, 0.1 mmol/L) was used to block the calcium current, resulting in an increased action potential response rate from 4.4±0.9% at baseline to 68±17% after Cd (n=6, P=0.01) (Figure 2B). Cd inhibition of slow AHPs was fully reversible.

Adenosine Receptor Subtype
To determine the adenosine receptor subtype responsible for blocking the slow AHPs, the adenosine A₁-receptor agonist 2-chloro-N6-cyclopentyladenosine (CCPA, 100 nmol/L) was administered to 7 cells with slow AHPs. After 3 minutes of exposure to CCPA, there was no detectable attenuation of slow AHPs or significant increase in action potential response rate (4.4±2.6% versus 9.7±4.5%, P=NS) (Figure 3).

In 8 cells with slow AHPs, adenosine was administered in the presence of the selective A₁-receptor blocker 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, 5 μmol/L). Slow AHPs and action potential response rate remained similar to the response rate in the presence of adenosine alone in the same neurons (Figure 4).

The adenosine A₁-receptor agonist 5′-([N-ethylcarboxamido] adenosine (NECA, 10 μmol/L) was administered to 4 cells with slow AHPs. NECA decreased the delayed AHP amplitude and increased action potential response rate (Figure 5), with a similar time course as adenosine from 2.8±0.9% at baseline to 37±11% after NECA (P=0.02). This increased excitability was reversible (washout rate, 4.8±1.1%).

In 6 cells with slow AHPs, adenosine was administered in the presence of the selective A₂-receptor blocker 4-[2-(7-amino-2-(3-furyl)-1(2,4)triazolo[2,3-a][1,3,5]triazin-5-ylaminio)ethyl]phenol (ZM 241385, 10 nmol/L). The diminution of slow AHPs and the increase in action potential

Figure 3. CCPA (100 nmol/L, A₁-adenosine receptor agonist) did not increase action potential response rate. Baseline action potential response rate was 4.4±2.6%, which, after CCPA, was 9.7±4.5% (P=NS) and during washout was 7.6±3.3% (P=NS).

Figure 4. Adenosine augmentation of action potential response rate is not blocked in presence of DPCPX (5 μmol/L, A₁-adenosine receptor antagonist). Eight cells in which adenosine reversibly increased action potential response rate (baseline, 3±1%; after adenosine, 24±9%; P<0.02) were then treated with adenosine in presence of DPCPX. Adenosine/DPCPX reversibly increased action potential response rate in these cells to similar degree as adenosine alone (baseline, 3.7±0.9%; after adenosine/DPCPX, 21±8%; P<0.02).
response rate induced by adenosine in these cells was abolished in the presence of ZM 241385 (Figure 6).

**Effects of Adenosine on I_{Ca}**

To determine if adenosine partially blocked I_{Ca} in rabbit vagal afferent neurons, as we reported previously in rat vagal afferent neurons,7 a total of 20 cells were studied in the whole-cell voltage-clamp mode. I_{Ca} was completely blocked by the nonspecific I_{Ca} blocker Cd (0.1 mm), but adenosine did not inhibit inward I_{Ca} in 20 cells tested (Figure 7).

**Discussion**

The major findings of this study are (1) adenosine blocks slow AHPs when they are present on vagal afferent neurons, markedly enhancing excitability; (2) this effect is mediated through the adenosine A_{2A} receptor; and (3) in rabbit nodose ganglion cells studied here, in contrast to rat nodose ganglion cells, adenosine does not inhibit I_{Ca}. The likely mechanism by which slow AHPs are suppressed is through enhanced cAMP production after A_{2A} receptor stimulation rather than suppression of I_{Ca}.

During muscle ischemia, a number of compounds are released that have been shown to be neuroexcitatory in afferent neurons, including lactic acid, arachidonic acid, and potassium ions.6,13 When adenosine is administered directly to isolated sensory neurons, it does not stimulate action potentials.6,7 Our results help clarify adenosine’s role as a neuroexcitatory agent. Adenosine does not appear to directly trigger neuroexcitation but by attenuating slow AHPs enables vagal afferent neurons to fire rapidly and repeatedly in the presence of other neuroexcitatory stimulants. Therefore, during muscle ischemia, when many neuroexcitatory compounds are released, adenosine may facilitate expression of this neuroexcitation.

In rabbit nodose ganglion cells, this neuroexcitatory role appears to be mediated by the adenosine A_{2A} receptor. NECA, a selective A_{2A} receptor agonist, reproduced the effects of adenosine, whereas CCPA, a selective adenosine A_{1} agonist, did not. In addition, the selective A_{2A}-adenosine antagonist ZM 241385 but not the A_{1}-adenosine antagonist DPCPX abolished augmentation by adenosine of the action potential response rate. In these experiments, our findings suggest that the most likely mechanism by which adenosine attenuates the delayed AHP is through an increase in cAMP. Adenosine A_{2A} receptors have been shown to increase cAMP through adenylyl cyclase, whereas adenosine A_{1} receptors inhibit adenylyl cyclase.14 Slow AHPs present on hippocampal CA3 neurons and on nodose ganglion neurons are directly inhibited by cAMP,8,15 which we confirmed in our preparation with forskolin, a direct activator of adenylate cyclase. Forskolin both attenuated slow AHPs and augmented action potential response rate.8 An alternative mechanism for enhanced neuroexcitability is suppression of I_{Ca} by adenosine, leading to a smaller rise in intracellular calcium during stimulation, less activation of IK(Ca), and suppression of the action potential response rate by adenosine in the presence of ZM 241385 (Figure 6).
slow AHP. We showed that this mechanism is generally plausible by blocking ICa with cadmium, which suppressed slow AHPs and enhanced excitability as postulated. However, in the rabbit nodose ganglion cells, adenosine had no effect on ICa, eliminating this as a possible contributing mechanism. This contrasts with our previous observations in rat nodose ganglion cells, in which adenosine inhibited ICa by 50% in approximately two thirds of cells. Interestingly, rat nodose ganglion cells do not exhibit slow AHPs, so other mechanisms of enhancing neuroexcitability may be important.

Limitations
Because adenosine had no effect on ICa, our findings imply by exclusion that adenosine eliminates slow AHPs by increasing cAMP, consistent with the properties of adenosine A2A receptors, which are coupled positively to cAMP production. Further studies are needed to directly test this hypothesis and to determine whether the effects of cAMP are mediated by protein kinase A or other cAMP-dependent signaling pathways. These investigations are currently ongoing in our laboratory. Because slow AHPs have been detected in a substantial proportion of vagal afferent neurons in several but not all species, the direct relevance of this work to humans, in whom the proportion of vagal afferents that have slow AHPs is unknown, remains to be determined.

Summary and Implications
These data are consistent with a neuroexcitatory role for adenosine, acting on the adenosine A2A receptor in rabbit nodose ganglion cells. Vagal afferent neurons modulate ischemic pain, including ischemic cardiac pain, as well as mediate systemic reflexes. Our findings may have implications for understanding the pathophysiology of mechanisms of pain during cardiac ischemia. It is unknown if adenosine has similar neuroexcitatory effects on sympathetic afferent neurons, but these studies are ongoing in our laboratory.

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References
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