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Adenosine Release Evoked by Short Electrical Stimulations in Striatal Brain Slices Is Primarily Activity Dependent

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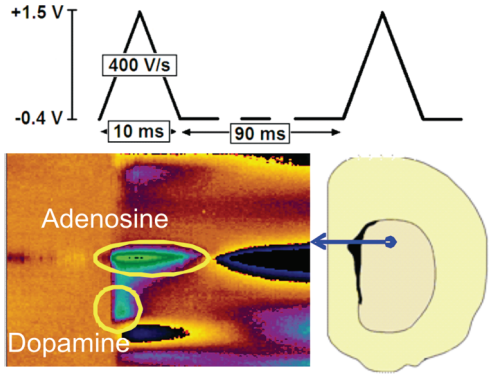
Abstract



Adenosine is an important neuromodulator in the brain. Traditionally, adenosine is thought to arise in the extracellular space by either an extracellular mechanism, where it is formed outside the cell by the breakdown of released ATP, or an intracellular mechanism, where adenosine made inside the cell is transported out. Recently, a third mechanism of activity dependent adenosine release has also been proposed. Here, we used fast-scan cyclic voltammetry to compare the time course and mechanism of adenosine formation evoked by either low- or high-frequency stimulations in striatal rat brain slices. Low-frequency stimulations (5 pulses at 10 Hz) resulted in an average adenosine efflux of $0.22 \pm 0.02\,\mu$M, while high-frequency stimulations (5 pulses, 60 Hz) evoked $0.36 \pm 0.04\,\mu$M. Blocking intracellular formation by inhibiting adenosine transporters with *S*-(4-nitrobenzyl)-6-thioinosine (NBTI) or propentofylline did not decrease release for either frequency, indicating that the release was not due to the intracellular mechanism. Blocking extracellular formation with ARL-67156 reduced low-frequency release about 60%, but did not affect high-frequency release. Both low- and high-frequency stimulated release were almost completely blocked by the removal of calcium, indicating activity dependence. Reducing dopamine efflux did not affect adenosine release but inhibiting ionotropic glutamate receptors did, indicating that adenosine release is dependent on downstream effects of glutamate. Therefore, adenosine release after short, high-frequency physiological stimulations is independent of transporter activity or ATP metabolism and may be due to the direct release of adenosine after glutamate receptor activation.

This experiment was conceived of by both Megan L. Pajski and B. Jill Venton. Research and data collection were performed by Megan L. Pajski. Manuscript writing and review were performed by Megan L. Pajski and B. Jill Venton.

Keywords: Caudate-putamen, carbon-fiber microelectrode, propentofylline, ARL 67156, reserpine, electrical stimulation

As a major neuromodulator and metabolite, adenosine modulates neuronal excitability, regulates blood flow, and plays an important role in controlling the energy supply in the brain (*1*−*4*). Four adenosine receptors with a wide range of affinities for adenosine allow careful regulation of its modulatory effects (*5*, *6*). Low concentrations of adenosine are inhibitory while high concentrations increase neuronal excitability by modifying the activity and availability of other receptor types, especially GABA and dopamine receptors (*3*, *4*, *6*, *7*). Adenosine plays a protective role against neuronal damage (*3*, *4*, *7*); therefore, its release has been studied under a variety of conditions, including after electrical stimulation, increased potassium levels, hypoxia, hypoglycemia, and ischemia (*2*).

Most studies have attributed extracellular adenosine formation to either an extracellular mechanism, where it is formed outside the cell, or an intracellular mechanism, where adenosine is formed inside the cell and transported out. Extracellularly, adenosine triphosphate (ATP) is metabolized by ATPase to adenosine monophosphate (AMP), which is converted to adenosine by 5′-ectonucleotidases. For intracellular formation, adenosine is formed from AMP after nucleotide degradation or from *s*-adenosyl homocysteine (SAH) (*2*), though previous studies suggest that the SAH pathway is only a minor source of adenosine (*8*, *9*). Adenosine formed inside the cell can then be transported out through bidirectional nucleoside transporters (*3*). These mechanisms are elucidated using pharmacology experiments. The

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extracellular mechanism is inhibited using drugs such as α,β-methylene adenosine diphosphate (AOPCP), which inhibits 5′-ectonucleotidase, or ARL-67156, which inhibits ATPase. The intracellular mechanism is inhibited by pharmacological agents that act on the bidirectional nucleoside transporters such as propentofylline, *S*-(4-nitrobenzyl)-6-thioinosine (NBTI), or dilazep (*10*).

Much of the literature on transient evoked adenosine changes has focused on the mechanism of formation under pathological conditions. While most studies suggest that the intracellular mechanism is more important, a few studies point to the extracellular mechanism (*8*, *9*, *11*−*17*). For example, adenosine release in response to metabolic poisoning due to toxin administration was reduced 80−90% by inhibiting adenosine transporters (*11*), implicating the intracellular mechanism. Similarly, many ischemia studies, which remove oxygen and glucose from the tissue, have also indicated a high intracellular contribution (*8*, *12*, *13*). In contrast, Phillis et al. only found a small contribution by the intracellular mechanism to ischemia-evoked adenosine (*12*), and Latini et al. found the extracellular mechanism to be the main contributor (*9*).

Similar disagreement exists about the mechanism of formation after electrical stimulation, which mimics the firing of neurons. Lloyd et al. showed that the primary source of electrically evoked adenosine was the intracellular mechanism (*8*), while the Cunha group found that this mechanism accounted for at most half of the released adenosine (*17*). Furthermore, the contribution of each mechanism was dependent on the frequency of stimulation. Using low-frequency electrical stimulations (1−10 Hz) (*18*) to mimic basal firing of neurons (*17*−*19*), 50% to 90% of adenosine efflux was from the intracellular mechanism, with extracellular formation playing a very minor role (*8*, *17*, *20*). During high-frequency stimulations that mimic burst firing rates (10−100 Hz) (*18*), the contribution of the intracellular mechanism decreased while the extracellular contribution increased (*21*). These studies applied minute-long stimulations which might not mimic normal firing because adenosine could only be measured every three to five minutes using radioactive detection or chromatography assays. In addition, the intracellular and extracellular mechanisms sometimes fail to account for 100% of adenosine formation, which could be attributed to drugs that are not completely effective or additional mechanisms of adenosine formation.

Another mechanism for adenosine formation which has been proposed is the direct release of adenosine in an activity-dependent manner (*2*, *22*, *23*). This mechanism can be tested by preventing action potentials with tetrodotoxin (TTX) or by removing calcium ions and preventing calcium-dependent exocytosis. While many studies have observed that adenosine release is calcium-independent (*24*−*28*), others suggest that with shorter, more physiological pulse trains, adenosine release is either partially or completely calcium-dependent (*2*, *17*, *20*, *29*, *30*). Therefore, this mechanism is still controversial. Activity-dependent adenosine release may result from downstream actions of other neurotransmitters or direct exocytosis of adenosine; however, adenosine has not yet been identified in vesicles.

In this study, we examined the mechanism of adenosine formation evoked by short trains of low- or high-frequency electrical stimulations. Subsecond adenosine changes were detected electrochemically using fast-scan cyclic voltammetry (FSCV) at 7 μm-diameter carbon-fiber microelectrodes (*31*, *32*), a method previously used to detect electrically stimulated release of adenosine in the caudate-putamen *in vivo* (*32*). The rat caudate-putamen was studied because it is an area rich in adenosine receptors (*33*, *34*). Electrical stimulations lasted less than half a second, making them more physiological than the longer duration stimuli in previous studies. We found that low-frequency stimulation elicits some adenosine release by the extracellular mechanism but that neither the extracellular nor the intracellular mechanisms accounted for the release observed after high-frequency stimulations. However, adenosine release after both stimulation frequencies was calcium-dependent. Further tests revealed that this activity-dependent adenosine release is dependent on glutamate receptor activation and thus is due to downstream effects of glutamate neurotransmission.

Results and Discussion

Spatial Distribution of Stimulated Adenosine Release

Changes in adenosine and dopamine can be measured simultaneously using fast-scan cyclic voltammetry. Figure 1 (top left) shows a color plot of an electrode calibration of 1 μM dopamine and adenosine, with time on the *x*-axis, applied voltage on the *y*-axis, and current in false color. Dopamine oxidizes at a potential of 0.6 V; therefore, the green signal in the lower half of all color plots is dopamine. The green signal in the middle of the color plot is from adenosine, whose main oxidation peak occurs at +1.5 V; thus, dopamine and adenosine are easily resolved.

Compounds with similar oxidation potentials can be difficult to discriminate; thus to confirm that adenosine is detected, we examined the sensitivity of our electrodes to possible interferents. We have previously shown that our electrode is twice as sensitive to adenosine as ATP (*35*), three times more sensitive than AMP, and does not detect inosine (*31*). Pharmacological data presented in this article show that ectonucleotidase inhibitors have no effect on high-frequency stimulated

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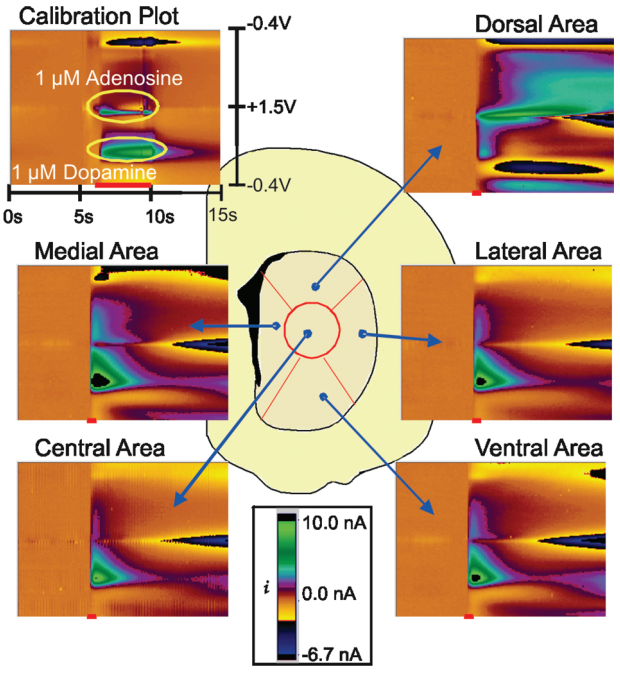

Figure 1. Spatial distribution of evoked adenosine in a striatal rat brain slice. A flow-cell calibration plot for a mixture of 1 μM dopamine and 1 μM adenosine is shown in the upper left-hand corner. In the calibration plot, the red line at the bottom of the plot represents the time that dopamine and adenosine were present. In the plots from slices, the red lines at the bottom represent the time of electrical stimulation (5 pulses at 60 Hz). All *in vitro* plots were taken with the same electrode in the same brain slice. The *x*-axis of each plot is time, with each file lasting 15 s. The *y*-axis plots the triangular FSCV waveform, starting at the bottom. The colors represent current, with all color plots scaled to the bar shown at the bottom. In order to visualize smaller signals, larger dopamine signals are occasionally off-scale (black). Although dopamine is detected throughout the caudate-putamen, adenosine is most readily detected in the dorsal caudate-putamen.

release, providing evidence that ATP is not the analyte being detected. Hypoxanthine detection was also examined because the Dale group has detected inosine and hypoxanthine after paired pulse stimulations (*36*). The sensitivity for hypoxanthine is two times lower than that for adenosine (Supporting Information, Figure 1), and the levels of hypoxanthine in the previous study are 2-fold smaller than adenosine release (*36*). Therefore, our electrodes are unlikely to be able to detect the small level of hypoxanthine that results from a short stimulation. In addition, hypoxanthine is a downstream metabolic product of adenosine; therefore, its appearance in the extracellular space is expected to be slow (*37*, *38*). Hydrogen peroxide could also be an interferent (*39*), although the oxidation peak occurs later than for adenosine, after the switching potential (Supporting Information, Figure 1). The sensitivity of the electrode is not as good for hydrogen peroxide, with detection limits around 6 μM, and there is no evidence for hydrogen peroxide levels this high after the stimulated release (*39*). Therefore, the measured signal is likely due to adenosine.

Spatial Distribution of Stimulated Adenosine Release

Stimulated adenosine release has been measured in the striatum using both long (*40*) and short (*32*) electrical stimulations but has not been characterized in striatal slices. The caudate-putamen was divided into medial, dorsal, lateral, ventral, and central areas. In Figure 1, an example color plot is shown for dopamine and adenosine release evoked by a 5 pulse, 60 Hz stimulation in each area. Color plots taken from brain slices are scaled the same in order to provide an idea of relative levels of release. Adenosine is only robustly detected in the dorsal area of the caudate-putamen, where the peak release in the plot corresponds to 0.24 μM adenosine. This peak occurs 2.8 s after the stimulation. Dopamine release in this same area is 0.20 μM and peaks 0.5 s after the stimulation. Adenosine signaling was slightly delayed from the start of the stimulation and lasted longer than the dopamine signaling, which is tightly controlled by the dopamine transporter. Interestingly, the area with the largest dopamine release, the medial caudate-putamen, showed only small adenosine release. Thus, the amounts of adenosine release and dopamine release did not appear to correlate. All further experiments were performed in the dorsal caudate-putamen to maximize adenosine measurements.

Other features are sometimes observed in the experimental data, such as blue and green areas at the bottom of the color plot, as well as blue areas that appear around the switching potentials, usually at 10 s or later and which grow larger as the file ends. These currents are not due to oxidation or reduction reactions of adenosine or dopamine but are changes in the background current caused by ionic changes after stimulation (*41*, *42*). Stimulation lengths were kept short to minimize these ionic changes.

Evoked adenosine was largest in the dorsal striatum, at a location similar to where our previous *in vivo* experiments were performed (*32*). This location dependence was not expected because physiology data show that adenosine receptors (*33*, *43*) and reuptake sites (*44*, *45*) are widely distributed throughout the striatum. However, only changes in adenosine levels were measured, and a basal level of adenosine may exist throughout the striatum. The location dependence of dopamine agrees with the work of Phillips and Stamford, who also observed greater dopamine release in the medial striatum than in the dorsal−lateral striatum (*46*). They found that dopamine release varied according to brain region and stimulation pattern because of the differential activation of calcium channels, and we hypothesize that regional variations in adenosine release could be controlled by similar effects.

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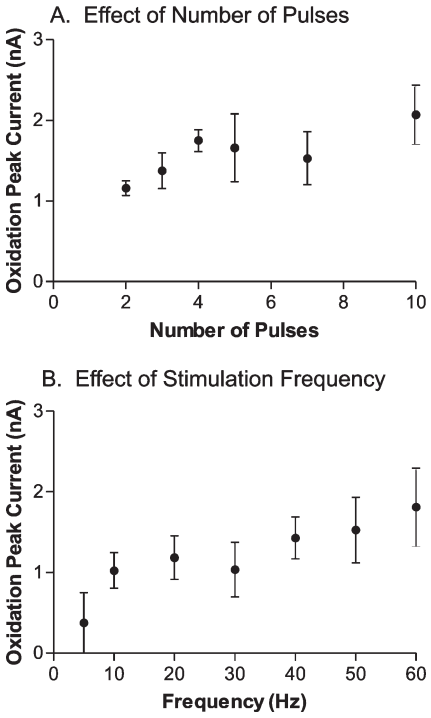

Figure 2. Effect of stimulation parameters on evoked adenosine. (A) Effect of increasing the number of pulses. Stimulation frequency was 60 Hz (n = 5 animals). (B) Effect of stimulation frequency. Five pulses were used (n = 5 animals).

Effect of Different Stimulation Parameters

Previous studies of adenosine release stimulated slices for minutes at a time (*29*, *30*), which might simulate a pathological event, and found that adenosine efflux varied with frequency and duration of stimulation. We examined short stimulation trains that are more consistent with natural firing patterns of neurons and varied the number of stimulation pulses and frequency. The stimulation parameters were adapted from stimulations previously used to evoke dopamine release (*47*, *48*).

First, we varied the number of stimulation pulses, applying 1, 2, 3, 4, 5, 7, or 10 pulses at a constant frequency of 60 Hz (Figure 2A). Oxidation peak current increased with increasing number of pulses. No adenosine was detected with one pulse stimulations, and adenosine was difficult to detect with two pulses. Larger numbers of pulses increased the likelihood that the tissue would show a large change in background current following stimulation, presumably from ionic changes. Generally, more than 10 pulses resulted in background changes that interfered with adenosine detection. Therefore, 5 pulse duration stimulation trains were chosen because they elicited a large oxidation peak current while minimizing extraneous background current changes.

Next, the effect of stimulation frequency was tested. Using a 5-pulse stimulation train, stimulation frequencies of 5, 10, 20, 30, 40, 50, and 60 Hz were applied (Figure 2B). Peak oxidation current increased with increasing frequency.

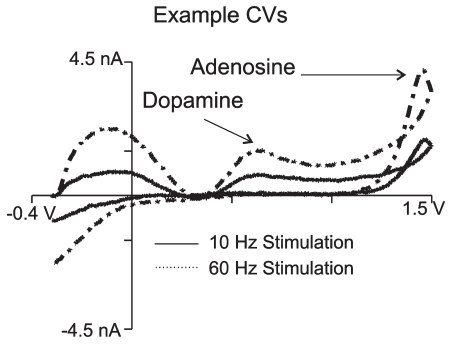

Figure 3. Low- vs high-frequency stimulated adenosine release. Example cyclic voltammograms (CVs) with dopamine (peak seen at 0.6 V) and adenosine (peak seen at 1.5 V) release are shown for 10 (low-frequency) and 60 (high-frequency) Hz stimulations. CVs were collected at the time of maximum adenosine release, corresponding to 0.7 s after stimulation at 10 Hz, and 0.9 s after stimulation at 60 Hz. The currents for both dopamine and adenosine are greater for 60 Hz stimulation.

Adenosine was more difficult to detect with lower frequencies, and reliable signals were not always observed at 5 Hz. A study by Pedata et al. also found that higher frequencies were needed, as adenosine could not be detected at frequencies less than 2.5 Hz using 5 min pulse trains (*29*). Therefore, 10 Hz was chosen for low-frequency stimulation, and 60 Hz was chosen for high-frequency stimulation.

Basal dopamine neuron firing is between 3 and 10 Hz, depending on where the neurons are located (*49*), and the 10 Hz chosen for low frequency stimulations is within this range. Burst firing rates for dopamine neurons are around 50 Hz; therefore, 60 Hz is similar to burst firing rates (*50*, *51*). *In vivo*, 10 and 60 Hz stimulations evoke very different patterns of dopamine, with 10 Hz exhibiting steady-state release, while 60 Hz release is peak shaped (*51*). While dopamine release in slices is typically evoked with a single pulse (*47*, *48*), adenosine release was not detected in our study with 1 pulse. Similarly, previous studies that identified activity-dependent adenosine release in the hippocampus (*23*, *52*) and cerebellum (*22*) used short pulse trains, and not single pulse stimulations.

Figure 3 shows sample cyclic voltammograms (CVs) for low (10 Hz) and high (60 Hz) frequency stimulations. The peaks for both dopamine (at 0.6 V) and adenosine (at 1.5 V) are larger with the 60 Hz stimulation. Table 1 compares average concentrations detected with the two frequencies for both adenosine and dopamine. Stimulated adenosine release was 50% greater with 60 Hz compared to 10 Hz stimulations, while dopamine release was 30% greater with the high-frequency stimulation. Thus, both adenosine and dopamine release were frequency-dependent.

Mechanisms of Adenosine Formation

The two main mechanisms proposed for adenosine formation are the extracellular mechanism, where

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Table 1. Stimulated Adenosine and Dopamine Release[a]

	peak evoked concentration (μM)	
stimulation frequency	adenosine	dopamine
10 Hz (5 pulses)	0.22 ± 0.02	0.13 ± 0.01
60 Hz (5 pulses)	0.36 ± 0.04	0.17 ± 0.02
p -value	0.0019**	0.1055
n	49	49

[a] p-Values (comparing 10 to 60 Hz stimulations): ** = $p < 0.01$.

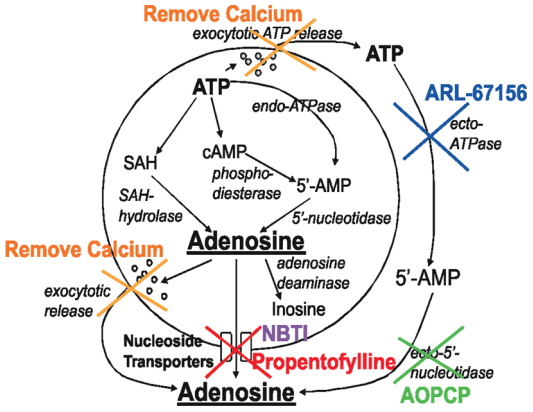

Figure 4. Sources of adenosine. There are three main mechanisms of adenosine formation. (1) The extracellular mechanism, where adenosine is formed by the breakdown of released ATP. This mechanism is inhibited by ARL-67156 (blue) and AOPCP (green). (2) The intracellular mechanism, where adenosine is formed inside the cell by the breakdown of ATP and then transported out through bidirectional nucleoside transporters. This mechanism is inhibited by propentofylline (red) and NBTI (purple). (3) An activity-dependent, exocytotic mechanism, where adenosine is formed inside the cell and then released into the extracellular space via vesicles. This mechanism is inhibited by the removal of calcium ions (orange).

adenosine is formed in the extracellular space, and the intracellular mechanism, where adenosine is formed inside the cell and transported out. A third possible mechanism is activity-dependent release, where adenosine formed inside the cell is directly released in an activity-dependent manner, independent of transporter activity (*2*, *22*, *23*). This mechanism is controversial because adenosine has not been identified in vesicles. Figure 4 shows a schematic of adenosine formation methods.

To test the mechanism of formation, pharmacological tests were performed. For all experiments, two baseline stimulations were performed 15 min apart, then the slice was perfused with a drug in aCSF for 30 min and another stimulation performed. Figure 5 shows that when saline was added to the aCSF as a control, the signal did not significantly change. The y-axis is the percentage of predrug stimulation, which is calculated in each animal by dividing the signal detected in the presence of drug by the predrug adenosine signal. Thus,

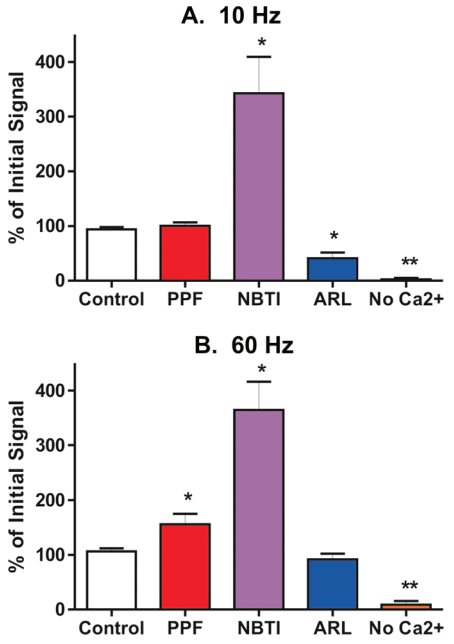

Figure 5. Effect of drugs on stimulated adenosine release. Values are plotted as percentages of the predrug response. Values are corrected for drug effects on electrode sensitivity for adenosine. (A) Ten Hertz stimulations (5 pulses). The control column shows that the addition of saline to the perfusing aCSF did not affect the adenosine signal. Propentofylline (PPF, 50 μM, red) did not affect adenosine release; however, *S*-(4-nitrobenzylthioinosine) (NBTI, 10 μM, purple) significantly increased adenosine levels, probably through the prevention of reuptake. ARL-67156 (ARL, 50 μM, blue) significantly decreased adenosine release. Removing calcium ions (no Ca^{2+}, plus 1 mM EDTA, orange) from the buffer greatly attenuated release. (B) Sixty Hertz stimulations (5 pulses). Both propentofylline and NBTI significantly increased adenosine levels, indicating that the intracellular mechanism controls reuptake, but not the release of adenosine. ARL-67156 had no effect on adenosine release; however, removing calcium ions from the aCSF significantly reduced the amount of adenosine detected. Thus, for both frequencies, adenosine release is calcium-dependent. For both frequencies, control, $n = 3$; PPF, $n = 6$; NBTI, $n = 5$; ARL, $n = 5$; no Ca^{2+}, $n = 5$. Significance indicators are shown for paired t tests performed between corresponding pre- and postdrug pairs. * $p < 0.05$; ** $p < 0.01$.

these values represent the change in adenosine levels due to the drug. Paired t tests were used to compare evoked concentrations before and after the drug in the same slice to test for significant changes.

In order to test the intracellular mechanism of formation, two different nucleoside transporter inhibitors were examined: propentofylline (PPF, 50 μM) or *S*-(4-nitrobenzyl)-6-thioinosine (NBTI, 10 μM) (Figure 4). These concentrations have been previously shown to slow down release or reuptake of adenosine in brain slices, demonstrating that they are effective transporter blockers (*10*, *17*). Figure 5 illustrates the effects of PPF and NBTI on 10 Hz (Figure 5A) and 60 Hz stimulations (Figure 5B). In flow-cell calibration experiments, the presence of propentofylline or NBTI reduces the sensitivity of the carbon-fiber microelectrodes for adenosine.

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To control for these effects, the data were corrected for the decrease in electrode sensitivity. With low-frequency stimulations, NBTI increased adenosine release significantly, about 3-fold, from 0.16 ± 0.02 μM to 0.53 ± 0.07 μM ($p < 0.05$). Propentofylline, however, had no effect. With high-frequency stimulations, both drugs increased adenosine levels. Adenosine increased from 0.35 ± 0.05 μM to 0.6 ± 0.1 μM with propentofylline ($p < 0.05$) from 0.42 ± 0.06 μM to 1.6 ± 0.4 μM with NBTI ($p < 0.05$). Because adenosine release did not decrease, the intracellular mechanism is not responsible for the formation of adenosine. The transporters also transport excess extracellular adenosine back into cells; therefore, an increase in concentration after the addition of transporter inhibitors indicates that with short electrical stimulations, the transporters are active in controlling reuptake. The difference in effects between the two drugs may be due to other properties of propentofylline. For example, propentofylline can act on A1 receptors, leading to inhibition of the cyclic AMP degradation (*53*), which could affect downstream cellular activity, including ATP release or adenosine concentration gradients. These results indicate that the intracellular mechanism is not responsible for adenosine evoked by short trains of low- or high-frequency stimulations.

To test the extracellular mechanism, ARL-67156 (ARL, 50 μM) was used to inhibit ATP breakdown. ARL-67156 inhibits the action of ATPase, preventing the breakdown of ATP into ADP and AMP (Figure 4). The dose of 50 μM ARL-67156 has been shown to potentiate the actions of adenosine receptor antagonists by preventing the formation of adenosine in slices (*54*). Using low-frequency stimulation, ARL-67156 significantly decreased adenosine efflux about 60%, from 0.13 ± 0.04 μM to 0.05 ± 0.02 μM ($p < 0.01$) (Figure 5A). However, it had no effect on high-frequency stimulation (Figure 5B). Although ARL-67156 is one of the more selective inhibitors of ATP breakdown, it may not be 100% effective at blocking ATP metabolism (*55*). Therefore, we tested some additional slices with a combination of 50 μM ARL-67156 and 100 μM adenosine-5′-(α,β-methylene) diphosphate (AOPCP). AOPCP is an inhibitor of ectonucleotidase, thus inhibiting the breakdown of AMP into adenosine (Figure 4). Together, ARL-67156 and AOPCP would inhibit both of the main steps of ATP breakdown to adenosine. The combination of ARL-67156 and AOPCP had no effect on adenosine release elicited by high-frequency stimulations (Supporting Information, Figure 2), indicating that the lack of effect of ARL-67156 is not due to the ineffective blocking of ATP metabolism. Thus, while the extracellular mechanism may be responsible for a portion of low-frequency stimulated release, it does not account for adenosine evoked by high-frequency stimulation.

By definition, activity-dependent release is dependent on action potentials and calcium influx (*22*, *23*, *56*). In order to block activity-dependent release of adenosine, Ca^{2+} was removed from the buffer and 1 mM EDTA added to chelate any remaining calcium in the slices (*57*) and block calcium-dependent exocytosis (*58*). Removing calcium ions almost completely attenuated adenosine and dopamine release for both 10 Hz ($p < 0.01$) and 60 Hz ($p < 0.01$) stimulation frequencies (Figure 5). Because EDTA chelates Mg^{2+} as well as calcium ions, a few slices were perfused instead with 1 mM EGTA, which preferentially chelates Ca^{2+} (Supporting Information, Figure 2). Slices respond to EGTA similarly to EDTA as dopamine and adenosine release still decreased to zero. These results indicate that adenosine formation for both stimulation frequencies is primarily activity-dependent.

In the literature, most studies attribute extracellular adenosine release to the intracellular and extracellular mechanisms, although there is disagreement as to the relative significance of the two mechanisms (*8*, *9*, *11*−*17*). For instance, Masino et al. showed that temperature-dependent increases in cellular activity caused adenosine release that was largely due to the intracellular mechanism (*15*), but MacDonald and White found the opposite to be the case when veratridine was used to increase cellular activity (*16*). Lloyd et al. found that 70−85% of electrically evoked adenosine was due to the intracellular mechanism (*8*), but the Cunha group found only a 50% contribution (*17*). The Cunha study also showed that the contribution of the intracellular mechanism is dependent on the frequency of stimulation, with higher frequencies producing less adenosine by the intracellular mechanism (*17*).

Contrary to what past studies have shown, our data indicates that the intracellular mechanism is not responsible for adenosine released by short electrical stimulations. This could be due to the duration of the stimulations, as our stimulations lasted less than a second, and previous studies used stimulations lasting several minutes. Under stress, increased ATP metabolism may lead to increased intracellular adenosine, which is then removed to the extracellular space by the nucleoside transporters. Subsecond stimulations may not represent a pathological situation that produces an excess of adenosine due to cellular activity, leaving the intracellular gradient unchanged. Therefore, the intracellular mechanism would be less important for short stimulations. The extracellular mechanism is a significant source of adenosine for low-frequency release but not for high-frequency release, which indicates that neither of the traditional mechanisms are responsible for high-frequency release. Thus, alternative mechanisms such as activity dependence were explored.

Both the low- and high-frequency stimulations produced adenosine release that was primarily activity-dependent.

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For low-frequency stimulations, the portion of the release that was due to the extracellular mechanism (exocytotic release of ATP) was expected to be activity-dependent. Complete block of low-frequency release indicates that the portion of release that was not affected by ARL-67156 was also activity-dependent. High-frequency release was also activity-dependent; however, due to the lack of response to ARL-67156 with high-frequency release, this calcium dependence cannot be attributed to the extracellular mechanism. The difference in mechanisms with different stimulation frequencies may be due to preferential activation of ion channels at certain frequencies. For example, Phillips and Stamford found that different calcium channels are active in the striatum during low-frequency stimulation than during high-frequency stimulation (*46*). Future pharmacology studies could determine whether differential calcium channel activation leads to the frequency-dependent patterns of adenosine release.

Effect of Dopamine and Glutamate Release on Adenosine Release

The decrease in the evoked adenosine signal after removing calcium suggested an activity-dependent component to adenosine release not fully accounted for by the traditional intracellular and extracellular mechanisms. Many studies have found partially or completely activity-dependent adenosine release, most of them utilizing short trains of stimuli to elicit release, which may represent nonpathological stimulations (*16*, *22*, *23*, *52*). As described in a review by Wall and Dale, the phrase "activity-dependent" can refer to multiple methods of chemical release (*56*). First, cellular stimulation may cause the direct vesicular release of a precursor, which is then metabolized into the compound (case 1). Second, cellular stimulation may cause the direct vesicular release of some neurotransmitter, which leads to downstream chemical stimulation of another cell, which then releases adenosine (case 2). Third, adenosine itself may be directly released from the stimulated cell by exocytosis (case 3). Case 3 is difficult to test directly, but cases 1 and 2 can be pharmacologically probed.

Case 1 is the extracellular mechanism that we tested, with ATP being the precursor. ARL-67156 is a competitive inhibitor of ecto-ATPase, and while large concentrations of ATP in the extracellular space can compete with the drug, the concentration applied was more than 150 times the K_i (*59*). In addition, adding a second ATP breakdown inhibitor, AOPCP, failed to increase the effect on adenosine release. Therefore, while ATP release may account for some (up to 60%) of the activity-dependent low-frequency release, it is unlikely to be the major source of adenosine for high-frequency release.

To test case 2, the downstream effects of the neurotransmitters dopamine and glutamate were blocked because they are the primary neurotransmitters released in the striatum. In our experiment, adenosine release is delayed from the stimulation by approximately half a second; thus, downstream actions of another neurotransmitter are a reasonable hypothesis. Dopamine release was decreased using reserpine, a vesicular monoamine transporter (VMAT) inhibitor that inhibits vesicular packaging and consequently vesicular release of dopamine, serotonin, and norepinephrine (*60*). Slices were perfused with reserpine for two hours, since reserpine is known to be slow acting (*61*), and stimulations were repeated every thirty minutes (Figure 6). Dopamine release was eliminated two hours after reserpine, while adenosine release remained constant for both stimulation frequencies. Therefore, adenosine release is not correlated with dopamine release and is not a downstream effect of its release. Our spatial distribution data (Figure 1) also showed that adenosine and dopamine release were not correlated as the areas of strongest dopamine release did not show robust adenosine release.

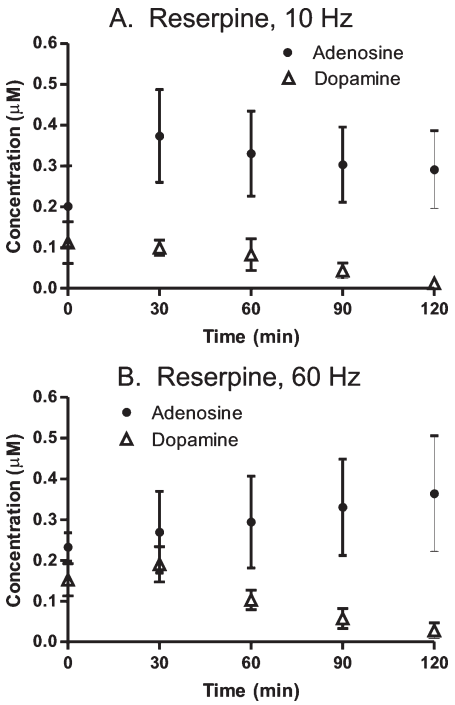

Figure 6. Effects of reserpine (10 μM) on adenosine (black dots) and dopamine (open triangles) release. Slices were perfused with reserpine for two hours, with stimulations being made every thirty minutes. (A) Low-frequency stimulation (n = 5). Reserpine decreased the amount of dopamine released but did not decrease adenosine release. (B) High-frequency stimulation (n = 5). Reserpine decreased dopamine release over time but had little effect on adenosine release.

Glutamate is also a major neurotransmitter in the striatum, and local electrical stimulations in slices would be expected to elicit glutamate release as well. Downstream effects mediated by ionotropic glutamate receptors

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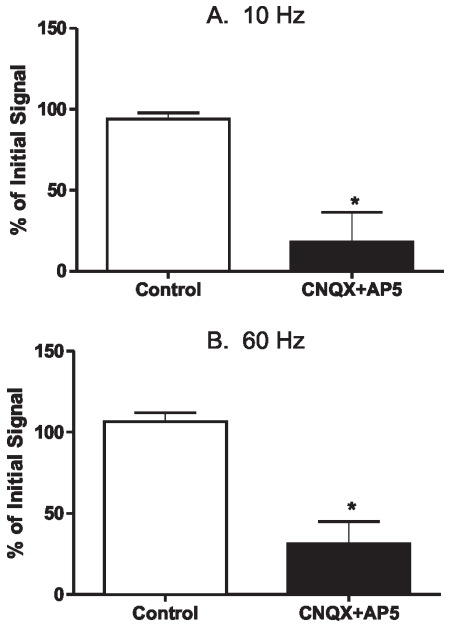

Figure 7. Effect of glutamate receptor antagonists. Iontotropic glutamate receptors were blocked with 10 μM CNQX (AMPA inhibitor) + 100 μM AP5 (NMDA inhibitor). (A) Ten Hertz stimulations (5 pulses). The addition of CNQX + AP5 (black) significantly reduced adenosine release to 18% of predrug release ($p < 0.05$); $n = 3$ slices. (B) Sixty Hertz stimulations (5 pulses). The addition of CNQX + AP5 (black) significantly reduced adenosine release to 31% ($p < 0.05$); $n = 5$ slices. Values are corrected for drug effects on electrode sensitivity for adenosine.

were inhibited using a combination of 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), an AMPA receptor inhibitor, and 100 μM D-(−)-2-amino-5-phosphonopentanoic acid (AP5), an NMDA receptor inhibitor. These doses have also previously been shown to inhibit glutamate release in slices (*22*). Because CNQX and AP5 decreased the electrode sensitivity for adenosine, the measured currents were corrected to account for this decrease. For both stimulation frequencies, stimulated release was reduced after ionotropic glutamate receptor blockade (Figure 7). CNQX and AP5 decreased adenosine release to 18% of predrug values with 10 Hz stimulations, from 0.16 ± 0.07 μM to 0.05 ± 0.05 μM ($p < 0.05$). Because ATP breakdown (extracellular mechanism) accounted for 60% of low-frequency stimulated release, these data imply that some of the ATP release is a downstream effect of glutamate. ATP and glutamate are coreleased from vesicles; therefore, a decrease in glutamate signaling might also decrease ATP release. With 60 Hz stimulation frequencies, adenosine decreased to 31% of initial values, from a predrug value of 0.4 ± 0.1 μM to 0.11 ± 0.05 μM ($p < 0.05$). This indicates that a downstream effect of glutamate is the predominant mechanism for activity-dependent adenosine formation. Our data differ from activity-dependent adenosine release measured in the cerebellum, where downstream actions of glutamate and GABA were not found to affect adenosine release (*22*). Therefore, the mechanism may be dependent on brain region. Since glutamate is the primary excitatory neurotransmitter in the brain, correlated adenosine and glutamate release could be important in modulating postsynaptic cell excitation.

Adenosine release is a downstream effect of activity-dependent glutamate release; however, there is still the question of how adenosine is released into the extracellular space after glutamate receptor activation. Because glutamate release is activity-dependent, the mechanism of adenosine release itself does not necessarily have to be activity-dependent. However, for high-frequency stimulations, our pharmacology experiments eliminated the breakdown of ATP and the release of adenosine through nucleoside transporters. Though propentofylline and NBTI preferentially inhibit equilibrative nucleoside transporters over concentrative nucleoside transporters, concentrative nucleoside transporters are not widely distributed within the brain and are unlikely to be the source of adenosine (*62*, *63*). In addition, neither equilibrative nor concentrative transporters are known to be activity or calcium dependent (*62*, *63*). Gap-junction hemichannels on astrocytes are calcium-dependent sources of non-neuronal neurotransmitter release, but only glutamate and ATP have been found to be released in this manner (*64*, *65*). Activation of ionotropic glutamate receptors opens cation channels, which would depolarize cells and lead to action potentials. With no other transporters or channels known to release adenosine, this leaves open the possibility that adenosine is released by direct exocytotic adenosine release from the postsynaptic cell. Direct exocytosis of adenosine is controversial because adenosine has not been identified in vesicles. However, direct exocytotic release could be advantageous (*56*). Direct release of adenosine would enable an inhibitory feedback mechanism onto neurons via A1 receptors, modulating both the activated pathway and regional neurotransmission controlled by neighboring neurons. Therefore, adenosine release as a downstream action of glutamate would help modulate the excitatory role of glutamate.

Conclusions

In this study, we evaluated the time course and mechanism of adenosine release in striatal brain slices in response to short trains of electrical stimulations. While the traditional extracellular mechanism contributed to low-frequency stimulated release, high-frequency release was not caused by either the intracellular or extracellular mechanism. However, both low and high frequency release were inhibited by the removal of calcium, showing

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that in both cases release was activity-dependent. The activity dependence was not a downstream effect of dopamine but was dependent on glutamate receptor activation. Therefore, adenosine release evoked by short stimulations is caused by downstream effects of glutamate. This work shows that nonpathological stimulations produce adenosine release in a manner linked with neurotransmission and further studies of the mechanism of adenosine release will help to better understand the regulation of adenosine and the amount available to modulate neurotransmission.

Methods

Chemicals

Dopamine and adenosine were purchased from Sigma Aldrich (St. Louis, MO) and 10 mM stock solutions prepared in 0.1 M perchloric acid and diluted to 1 μM in aCSF on the day of the experiment. The artificial cerebral spinal fluid (aCSF) solution was 126 mM NaCl, 2.5 mM KCl, 1.2 mM NaH_2PO_4, 2.4 mM $CaCl_2$ (dehydrate), 1.2 mM $MgCl_2$ (hexahydrate), 25 mM $NaHCO_3$, 11 mM glucose, and 15 mM tris(hydroxymethyl) aminomethane, with the pH adjusted to 7.4. For calcium-free experiments, the aCSF solution was made exactly as before, only with $CaCl_2$ removed and 1 mM ethylenediaminetetraacetic acid (EDTA) or 1 mM ethylene glycol-bis(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) added. All buffer salts were from Fisher Scientific (Waltham, MA). Aqueous solutions were made using doubly deionized water (Millipore, Billerica, MA). Propentofylline, α,β-methylene adenosine diphosphate (AOPCP), EGTA, and reserpine were purchased from Sigma Aldrich; ARL-67156, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and D-(−)-2-amino-5-phosphonopentanoic acid (AP5) from Tocris Bioscience (Ellisville, MO); while EDTA and *S*-(4-nitrobenzyl)-thioinosine (NBTI) were from Fisher Scientific. Propentofylline, ARL-67156, AOPCP, EDTA, and EGTA were dissolved in aCSF. Stock solutions of 10 mM NBTI or 20 mM reserpine in dimethyl sulfoxide (DMSO, Fisher) were diluted to 10 μM in aCSF for use in slices. Drug solutions were normally used on the same day they were made but were occasionally frozen and used within 30 days.

Electrodes

Cylindrical carbon-fiber microelectrodes, about 50 μm in length, were made in house from T-650 carbon fibers (Cytec Engineering Materials, West Patterson, NJ) (*66*). Electrical connection was made by backfilling the capillary with 1 M potassium chloride. The electrodes were soaked in 2-propanol for at least 10 min prior to use. A chloridized silver wire was the reference electrode. Prior to use in a brain slice, carbon-fiber microelectrodes were calibrated in a flow injection apparatus, as described previously (*66*). Four-second sample injections of either 1 μM dopamine or 1 μM adenosine were used for calibrations. Fast-scan cyclic voltammograms were collected using a Chem-Clamp potentiostat (Dagan, Minneapolis, MN). The data acquisition software and hardware were the same as described by Heien et al. (*67*). A triangular waveform was applied to the electrode by first holding the electrode at a potential of −0.4 V, linearly ramping up to 1.5 V, then back down to −0.4 at 400 V/s at a repetition rate of 10 Hz. Prior to collecting any experimental data, electrodes were cycled with the waveform for 15 min in order to allow the background to stabilize. Current versus time plots were obtained by integrating the current in a 100 mV window centered around the oxidation peak for each cyclic voltammogram. Cyclic voltammograms were background-corrected by subtracting 10 averaged background scans taken immediately before the analyte was injected.

Animals and Slice Preparation

Adult, male Sprague−Dawley rats (250−350 g) purchased from Charles River were housed in a vivarium and given food and water *ab libitum*. All experiments were approved by the Animal Care and Use Committee of the University of Virginia.

To prepare slices, rats were first anesthetized with isoflurane (1 mL/100 g rat weight) in a desiccator. After beheading the rat, the brain was removed within 2 min and placed in ice-cold aCSF for 2 to 3 min. The aCSF was bubbled with a mixture of 95% oxygen and 5% carbon dioxide (carbogen) for at least 30 min prior to introduction of the brain tissue. Four hundred micrometer slices through the caudate-putamen were made using a vibratome (Leica VT1000S, Bannockburn, IL), with a slicing speed of 3 and a vibration frequency of 8. Slices from the caudate-putamen were then transferred to a beaker of room temperature aCSF bubbled with carbogen and allowed to recover for at least an hour prior to the experiment.

Brain Slice Experiment

Once a brain slice had recovered, it was transferred to a brain slice chamber. A multichannel, manual-control pump (Watson-Marlo 205U, Wilmington, MA) was used to flow room temperature aCSF over the brain slice at 1 mL/min. A carbon-fiber electrode was inserted so that the tip was approximately 75 μm below the top of the slice. The electrode was cycled for 30 min before data collection to allow the background to stabilize and the tissue to recover. The electrode position corresponded to approximately the following coordinates from bregma: +1.2 mm anterior−posterior, +2.1 to +2.3 mm mediolateral, and −4.2 to −4.5 mm dorsoventral. The bipolar stimulating electrode (Plastics One, Inc., Roanoke, VA), with 280 μm diameter wires spaced 800 μm apart, was placed so that the two prongs of the stimulating electrode and the working electrode formed a triangle. The stimulating electrode was about 500 μm from the working

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electrode. The slice was normally stimulated with pulse trains of 5 biphasic pulses, each 4 ms long (2 ms per phase) with an amplitude of 300 μA, at a frequency of 10 or 60 Hz, using a BSI-950 Biphasic Stimulus Isolator (Dagan). The tissue was allowed to recover for 15 min between stimulations. Adenosine was detected in approximately 25% of slices.

For electrode positioning experiments, the caudate-putamen was divided into five different areas where the working and stimulating electrodes were placed. All experiments were performed in the same brain slice, at a stimulation frequency of 60 Hz. The working electrode was allowed to equilibrate for 30 min each time it was moved to a different area of the brain slice.

For pulse number experiments, the brain slice was stimulated at a frequency of 60 Hz, and the pulse number was 1, 2, 3, 4, 5, 7, or 10, with 15 min between stimulations. For frequency experiments, the number of stimulations was fixed at 5 pulses, and the frequency was varied from 5, 10, 20, 30, 40, 50, and 60 Hz.

When testing pharmacological agents, two stimulations were initially performed, then the perfusion liquid was changed to aCSF containing drug for 30 min and a stimulation performed. Data were compared before and after the addition of drug in the same sample. To test for intracellular and extracellular mechanisms of release, slices were perfused with 50 μM propentofylline (PPF, K_i 20 μM) (*3*, *6*, *10*) or 10 μM *S*-(4-nitrobenzyl)-thioinosine (NBTI, K_i 1nM) (*68*), adenosine transporter inhibitors, or 50 μM of ARL-67156 (ARL, K_i 0.3 μM) (*54*, *59*, *69*), an ecto-ATPase inhibitor, respectively. Some slices were perfused with a combination of 50 μM ARL-67156 and 100 μM α,β-methylene adenosine diphosphate (AOPCP, K_i 5 nM) (*70*). Slices were perfused with calcium-free aCSF with 1 mM EDTA (to chelate any calcium ions remaining in the tissue; IC_{50} 50 μM) (*57*, *58*) or 1 mM EGTA (IC_{50} 46 μM) (*57*, *71*) to investigate activity-dependent release. To assess the dependency of adenosine release on dopamine release, slices were perfused with 10 μM reserpine (K_i < 1 nM) (*72*−*74*), a vesicular monoamine transporter inhibitor. In these experiments, slices were perfused with reserpine for 180 min and stimulations repeated every 30 min. To test the dependency of adenosine release on glutamate release, slices were perfused for 30 min with a combination of 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, K_i 1 μM) (*75*), an AMPA-receptor antagonist, and 100 μM D-(−)-2-amino-5-phosphonopentanoic acid (AP5, K_i 0.2 μM) (*76*), an NMDA receptor antagonist.

Electrode Calibrations and Drug Effects

For each drug or combination of drugs tested, a set of 6 electrodes was calibrated in the flow cell system with and without drug added to the aCSF to determine if the drugs had an effect on electrode sensitivity. On average, propentofylline reduced electrode sensitivity to adenosine to 66% of predrug sensitivity (a decrease of 34%), while NBTI decreased sensitivity to 32% of predrug sensitivity. Neither ARL-67156 nor a combination of ARL-67156 and AOPCP had a significant effect on the sensitivity toward adenosine. EDTA decreased sensitivity to adenosine by 3% (97% of predrug sensitivity). EGTA changed the shape of the adenosine CV but did not affect our conclusions. CNQX and AP5 decreased sensitivity to 18% of predrug sensitivity. In order to correct for drug effects on electrode sensitivity, adenosine concentrations postdrug were divided by the percentage value that the drug was found to decrease sensitivity in calibrations (i.e., propentofylline values were divided by 0.66).

Statistics

All values are reported as the mean ± standard error of the mean (SEM). Error bars are plotted as the SEM for values from *n* different slices. Experiments were repeated using slices from different rats, with a different electrode for each slice. Paired *t* tests were used to determine drug effects, comparing stimulated release before and after a drug in the same slice. All statistics were performed in GraphPad Prism (GraphPad Software, Inc., La Jolla, CA) and considered significant at the 95% confidence level.

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Supporting Information Available

Calibration current-voltage plots for 1 μM adenosine, 1 μM hypoxanthine, and 100 μM hydrogen peroxide (H_2O_2); effect of a combination of 50 μM ARL-67156 and 100 μM AOPCP, or 1 mM EGTA on adenosine release. This material is available free of charge via the Internet at http://pubs.acs.org.

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