Adenosine Receptor Antagonists Induce Persistent Bursting in the Rat Hippocampal CA3 Region Via an NMDA Receptor-Dependent Mechanism

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Thümmler, Susanne and Thomas V. Dunwiddie. Adenosine receptor antagonists induce persistent bursting in the rat hippocampal CA3 region via an NMDA receptor-dependent mechanism. J. Neurophysiol. 83: 1787–1795, 2000. Adenosine receptor antagonists initiate repetitive bursting activity in the CA3 region of hippocampal slices. Although some studies have suggested that this effect is irreversible, this has been difficult to establish because many adenosine antagonists wash out of brain slices extremely slowly. Furthermore the cellular mechanism that underlies persistent bursting is unknown. To resolve these issues, we studied the effects of nonselective (8-p-sulfophenyl-theophylline, 8SPT, 50–100 μM), A1-selective (8-cyclopentyl-1,3-dipropylxanthine, 100 nM; xanthine carboxylic acid congener, 200 nM), and A2A-selective (chlorostyryl-cafeine; 200 nM) adenosine antagonists in the CA3 region of rat hippocampal slices using extracellular recording. Superfusion with all of the adenosine antagonists except chlorostyryl-cafeine induced bursting, and the burst frequency after 30 min drug superfusion did not differ for the different antagonists. Most slices showed a period of rapid initial bursting, followed either by stable bursting at a lower frequency or a pattern of oscillating burst frequency. In either case, the bursting continued after drug washout. Virtually identical patterns of long-term bursting activity were observed when 8SPT was washed out or applied continuously. Control experiments using exogenous adenosine to characterize the persistence of 8SPT in tissue demonstrated >95% washout at 60 min, a time when nearly all slices still showed regular bursting activity. When the N-methyl-d-aspartate (NMDA) antagonists dl-2-amino-5-phosphonovaleric acid (AP5; 50 μM) or dizocilpine (10 μM) were applied before and during 8SPT superfusion, bursting occurred in the presence of the NMDA antagonists but did not persist once the 8SPT was washed out. AP5 had no effect on persistent bursting when applied after the initiation of spiking. The selective calcium/calmodulin-dependent protein kinase inhibitor 1-[N-O-bis-(5-isouquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine (KN-62; 3 μM), which has been shown to block NMDA receptor-dependent synaptic plasticity in the CA1 region, also significantly decreased the long-term effect of 8SPT. Thus adenosine antagonists initiate persistent spiking in the CA3 region; this activity does not depend on continued occupation of adenosine receptors by antagonists, and can be blocked by treatments that prevent NMDA receptor-dependent plasticity.

INTRODUCTION

Adenosine is a neuromodulator that has a largely depressant effect on neuronal activity in the CNS, and there is abundant evidence that there is a basal level of extracellular adenosine in the brain that exerts a tonic inhibitory effect on neuronal activity. The stimulatory effects of competitive antagonists of adenosine receptors such as caffeine and theophylline have been linked to the reversal of this tonic inhibitory effect.

Blockade of adenosine receptors with competitive antagonists produces at least three different types of effects in hippocampal neurons. First, there is an increase in the amplitude of synaptic responses at glutamatergic synapses such as the Schaffer collateral and commissural inputs to the CA1 region, which are tonically inhibited by presynaptic A1 receptors (Dunwiddie and Hoffer 1980). Second, there is a depolarization of the resting membrane potential, usually accompanied by an increase in input resistance, which corresponds to the closing of G-protein-coupled K+ channels that are activated tonically by postsynaptic A1 receptors (Alzheimer et al. 1993). Finally, antagonists can induce epileptiform activity, usually in the form of spontaneous bursting that appears to originate in the CA3 region (Alzheimer et al. 1989; Ault and Wang 1986; Ault et al. 1987); adenosine antagonists also increase the rate of spontaneous bursting induced by other agents such as bicuculline (Ault and Wang 1986; Dunwiddie 1980). Although the first two of these effects (the increase in glutamatergic synaptic responses, and the depolarization of the resting membrane potential) appear to be fully reversible, there are several reports that the bursting induced by adenosine receptor antagonism is irreversible, i.e., that once initiated, it persists after the washout of the antagonist (Alzheimer et al. 1989; Ault et al. 1987; Chesi and Stone 1997). It is unclear why most of the effects of antagonists are reversible, but the induction of epileptiform bursting is not. This issue is complicated by the fact that the agents that are most effective in inducing persistent bursting, such as 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), have subnanomolar affinities for the A1 receptor and wash out of the tissue very slowly. Thus it has been difficult to exclude the possibility that the persistent bursting simply reflects the continued presence of low concentrations of antagonist. This possibility receives further support from the observation that theophylline, which washes out of slices relatively quickly, initiates bursting that terminates promptly on washout (Ault and Wang 1986; Chesi and Stone 1997; but see Ault et al. 1987). Determining the mechanism underlying persistent effects has been complicated further by inconsistencies in the literature. These are likely to reflect differences in important
experimental variables related to interictal bursting, such as the use of different species (guinea pig, rat), different Ca$^{2+}$, Mg$^{2+}$, and K$^+$ concentrations in the medium, and the application of various adenosine antagonists over different time periods.

At present, at least three potential explanations have been proposed to account for the persistent bursting induced by some adenosine receptor antagonists and not others. The continued presence of low concentrations of lipophilic antagonists in the tissue due to limited drug washout might account for persistent effects; alternatively, selective A1 receptor antagonists such as DPCPX might induce irreversible effects, whereas antagonists such as theophylline that do not show selectivity for A1/A2 receptors do not, or blockade of adenosine receptors could under some conditions activate a second-messenger-mediated mechanism that leads to continued bursting. In terms of this last mechanism, previous studies have shown that a brief period of bursting induced by agents that do not interact with adenosine receptors can sometimes become persistent, reflecting the initiation of an N-methyl-d-aspartate (NMDA) receptor-dependent form of plasticity (Schneiderman et al. 1994). The present experiments were designed to examine this latter possibility, and to determine whether such a mechanism could explain the previously reported persistency of bursting induced by adenosine receptor antagonists.

METHODS

Male Sprague Dawley rats from 4 to 7 wk old were used for all experiments. Housing and treatment of all animals were in accordance with animal welfare protocols approved by the Institutional Animal Care And Use Committee. Animals were decapitated, and a Vibratome (Ted Pella) was used to prepare 400-μm-thick coronal slices of hippocampus. The slices were incubated in artificial cerebral fluid (ACSF) containing (in mM): 126 NaCl, 3.0 KCl, 1.5 MgCl$_2$, 2.4 CaCl$_2$, 1.2 NaH$_2$PO$_4$, 11 glucose, and 25.9 NaHCO$_3$. As in previous studies, this latter solution was used during experiments because spontaneous bursting rarely occurs in media containing the higher concentrations of divalent cations found in our normal ACSF. Extracellular recordings were made using glass pipettes pulled on a Flaming/Brown electrode puller (Sutter Instruments, Novato, CA) filled with 3 M NaCl. For recording spontaneous bursts, the recording electrode was placed under visual guidance in the stratum pyramidale of the CA3 region. A twisted polar nichrome wire electrode was placed as stimulation electrode in s. radiatum of the CA3 region and the placements of the recording electrode in s. radiatum of CA3, and the placements of the electrodes were adjusted so that large-amplitude population spikes could be recorded as an indication of slice viability and proper electrode positioning. The placement of the recording electrode remained unchanged for the duration of the experiment. Individual bursts were detected with a window discriminator, which was used to trigger data acquisition on a computer-based analysis program. For display and analysis purposes, the instantaneous burst frequency was defined as the reciprocal of the time since the preceding burst (i.e., a 2-s interburst interval would correspond to a frequency of 0.5 Hz). The average of all the frequencies during each 5-min period of time was averaged and used for subsequent analysis of changes in burst rate. For recordings of field excitatory postsynaptic potential (fEPSP) responses, the recording electrode was placed in s. radiatum of the CA1 region and the stimulation electrode in s. radiatum near the border of the CA1 and CA2 regions; stimuli were delivered at 15-s intervals. For delivery of drug solutions, stock solutions were made at 50–200 times the desired final concentration of drug, and a calibrated syringe pump (Razel) was used to add drugs directly to the superfusion system. In no case was the perfusion speed increased by >1% by drug addition.

All electrophysiological responses were recorded to computer disk for later analysis using software developed in our laboratory. Data were analyzed subsequently with Microsoft Excel and GraphPad Prism. Statistical analyses of drug effects were performed using the two-tailed unpaired or paired Student’s t-test with a $P < 0.05$ criterion for statistical significance, and the χ$^2$ or Fisher’s exact test were used for analysis of the proportion of slices showing bursting activity under different conditions. All summary data are reported as the means ± SE. Adenosine and dl-2-amino-5-phosphonovaleric acid (AP5) were purchased from Sigma. 8-(p-sulfophenyl)theophylline (8SPT), dicycline hydrochloride maleate, and 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) were purchased from Research Biochemicals International, and 1-[N-O-bis-(5-isouquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phe- nylpiperazine (KN-62) was purchased from Calbiochem. Xanthine carboxylic acid congener (8-[4-[(carboxymethyl)oxy]phenyl]-1,3-dipropylxanthine; XCC) was kindly provided by Dr. Kenneth A. Jacobson (Jacobson et al. 1986).

RESULTS

Adenosine A$_1$ receptor antagonists induce bursting in the CA3 region of rat hippocampus

Previous studies have reported that both nonselective (theophylline) (Ault and Wang 1986; Chesi and Stone 1997) and selective A1 receptor antagonists (DPCPX) (Alzheimer et al. 1989, 1993; but see Chesi and Stone 1997) induce repetitive bursting in the CA3 region of rat and guinea pig hippocampal slices. Because some of the differences in the results of these studies might be related to differing pharmacological specificities of the antagonists, in initial experiments we compared the effects of four adenosine receptor antagonists with varying receptor selectivity (Table 1) on bursting activity in rat hippocampal slices. We previously have found that DPCPX and the closely related cyclopentyl-theophylline require ≥30 min of superfusion to achieve equilibrium concentrations in hippocampal slices (Dunwiddie and Diao 1994; T. V. Dunwiddie and L. Diao, unpublished results); therefore all slices were superfused with antagonists for a minimum of 30 min. Of a total of 158 slices tested, 27/158 (17%) showed spontaneous bursts without any prior drug application and were subsequently not tested. In 74/131 (56%) slices, bursts were induced with DPCPX, 8SPT, or XCC, and in 57/131 (44%), no bursts could be induced with these antagonists. When tested alone,

<table>
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<tr>
<th>Antagonist</th>
<th>$A_{1}$ $K_{i}$, nM</th>
<th>$A_{2A}$ $K_{i}$, nM</th>
<th>$A_{2B}$ $K_{i}$, nM</th>
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<td>XCC</td>
<td>42–58°C</td>
<td>1130°C</td>
<td>68°C</td>
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<td>1280°C–4500°C</td>
<td>3320°C</td>
<td>6300°C</td>
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<td>DPCPX</td>
<td>0.5–8.06°C</td>
<td>260–529°C</td>
<td>1440°C</td>
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<tr>
<td>CSC</td>
<td>28,000°C</td>
<td>54°C</td>
<td>Not known</td>
</tr>
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XCC, 8-[4-[(carboxymethyl)oxy]phenyl]-1,3-dipropylxanthine; 8SPT, 8-p-sulphophenyltheophylline; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; CSC, chlorostyryl-caffeine.

* Jarvis et al. (1989); † Jacobson et al. (1986); ‡ Daly et al. (1985); § Kirk and Richardson (1995); ¶ Jacobson et al. (1993).
the nonselective antagonist 8SPT induced spiking in 32/54 (59%) slices; it was tested at both 50 and 100 μM with similar results (Table 2). The A<sub>1</sub> receptor-selective antagonist DPCPX and XCC induced bursting in approximately the same percentage of slices as was observed with 8SPT, but the A<sub>2A</sub>-selective antagonist chlorostyryl-caffeine (CSC) did not induce spiking (0/7 slices). CSC also did not appear to either inhibit or enhance the bursting induced by 100 μM 8SPT (Table 2) because the latency to onset and frequency of bursting in slices pretreated with CSC and then tested with 8SPT was virtually identical to what was seen in control slices. Thus these results confirm the conclusion suggested by previous studies (Alzheimer et al. 1993; Chesi and Stone 1997) that displacement of endogenous adenosine from A<sub>1</sub> receptors is necessary and sufficient to initiate bursting. The similarity of the effects of the A<sub>1</sub>-selective antagonists and 8SPT and the lack of effects of CSC suggest that A<sub>2A</sub> receptors do not modulate this activity.

Although all of the effective antagonists induced bursting in a qualitatively similar manner, the bursting induced by both concentrations of 8SPT occurred with a shorter mean latency than was observed with either XCC or DPCPX. On the basis of concentration and potency, a much more rapid response to DPCPX (∼200 times its K<sub>i</sub> for the A<sub>1</sub> receptor) relative to 8SPT (∼20 times its K<sub>i</sub>) might have been predicted, when in fact the opposite was observed (i.e., the response to 8SPT was significantly faster; P < 0.001). This suggests that the latency to the onset of spiking with these agents is more closely related to their physical properties (i.e., rate of diffusion into the brain slice) rather than interactions with the receptor, as has been noted in previous studies with these and other closely related antagonists (Dunwiddie and Diao 1994). Because 8SPT is much more water soluble than DPCPX, it diffuses into (and out of) the slice readily, whereas the diffusion of a lipid-soluble antagonist such as DPCPX may be retarded because it is essentially trapped by partitioning into cell membranes. Because XCC is somewhat more water soluble (i.e., less lipophilic) than DPCPX, it was hypothesized that it would wash in and out of slices more quickly than DPCPX; although there was a nonsignificant trend in this direction, the latency to onset of bursting with XCC was still slower than for 8SPT. In subsequent experiments, the more-water-soluble 8SPT was used because, in contrast to the selective A<sub>1</sub> antagonists, it could be washed in and out of brain slices relatively quickly. In addition, 8SPT normally is charged in aqueous solution and cannot cross cell membranes. Thus unlike the other antagonists, its effects must reflect actions at extracellular receptors, presumably for adenosine, and cannot involve actions at intracellular sites, such as inhibition of cyclic nucleotide phosphodiesterase.

The most common pattern of activity elicited by all of the antagonists was an initial peak in the rate of spontaneous bursting that occurred within the first few minutes of bursting, followed by a rapid decline in rate to a relatively stable level that was maintained throughout the period of drug superfusion (Figs. 1, A and C, 2A, and 5A). Once stable bursting was attained, the amplitude and duration of the bursts usually did not change markedly (Figs. 1, B and D, and 5B), but the frequency sometimes declined slowly over the course of the experiment. In the slices that showed stable bursting, the frequency of bursting during the period from 25 to 30 min after the onset of superfusion with the antagonists was not significantly different for any of the antagonists tested, and ranged from ∼0.15 to 0.2 Hz (Table 2). Thus all of the antagonists with substantial affinity for the A<sub>1</sub> receptor induced repetitive bursting in approximately the same fraction of slices, and their adenosine receptor selectivity or nonselectivity did not appear to affect the incidence or characteristics of the bursting.

In ∼15% of the slices, the pattern of bursting did not reach a stable level, but showed regular oscillations in the frequency of bursting (Figs. 2B and 6). The mean period of these oscillations was 4.4 ± 0.44 min, and in most cases, the amplitude of these oscillations declined over time. The occurrence of these oscillations did not appear to be related to the antagonist used to induce the bursting and was seen with each of the effective antagonists. Extreme oscillations in frequency were observed in 2/74 slices (e.g., Fig. 2C); in these slices, the burst frequency was taken to be the frequency of baseline activity before and after the episodes of high-frequency bursting.

### Persistence of repetitive bursting

As has been reported in previous studies (Alzheimer et al. 1989, 1993), bursting induced by DPCPX was essentially irreversible (>1 h with minimal decrement) even with extended washing. However, demonstrating washout of DPCPX is problematic because it is highly lipid soluble and equilibrates very slowly with brain slices. Furthermore the issue was complicated by the fact that the rate of bursting decreased over long periods of time (Fig. 1A) (see also Alzheimer et al. 1989, Fig. 1), and it was unclear whether this was due to slow washout of drug or to changes in the intrinsic rate of bursting that were independent of the presence of the antagonist in the slice. To resolve these issues, we characterized the persistent effects of 8SPT, which washes in and out of tissue more quickly than DPCPX, and used two approaches to demonstrate that the persistent bursting was not due to the continued presence of 8SPT in the slices. First, the rate at which 8SPT washed out of slices was determined experimentally. In this protocol (illustrated in Fig. 3A), the presence of 8SPT in the slice was
inferred based on its ability to antagonize the effects of a low concentration of adenosine (20 \( \mu M \)) on the fEPSP response evoked by synaptic stimulation. Slices were superfused with 8SPT for 30 min, and the subsequent washout of 8SPT was monitored by the return of the synaptic response to its inhibited baseline (Fig. 3A). After 60 min of 8SPT washout (in the continued presence of adenosine), the fEPSP amplitude returned to the original baseline (Fig. 3B), suggesting that 8SPT washout was complete within this time period. The actual time course of the washout of 8SPT is illustrated in Fig. 4 and confirms that there is no significant effect of 8SPT after 1 h of washout. In a second set of experiments, the repetitive bursting was compared in a group of slices that was superfused with 100 \( \mu M \) 8SPT for 30 min, followed by drug washout, and in a second group that was superfused continuously with 8SPT throughout the experiment. The results for both groups were virtually identical (Fig. 4), which suggested that the decline in bursting observed during a 60-min washout period was unrelated to washout because it occurred to a similar extent in slices continuously superfused with drug. These data confirm the conclusion of a previous study (Alzheimer et al. 1989) that

**FIG. 2. Patterns of repetitive bursting induced by 8SPT (nonselective) and 8-[4-((carboxymethyl)oxy)phenyl]-1,3-dipropylxanthine (XCC; A1 selective) adenosine receptor antagonists.** Three different patterns of repetitive bursting typically were observed. Pattern illustrated in A, which was most common, was an initial peak in the burst rate, followed by a fairly rapid decline to a stable level of firing. A somewhat lesser number of slices showed an initial peak, followed by a cyclical pattern of activity where the rate fluctuated with a period of 4–5 min (B). Amplitude of the oscillations normally decreased over time. Finally, a few slices showed a biphasic pattern of low frequency activity (~0.2 Hz), which was interspersed with periods of much faster bursting (C). There did not appear to be any significant relationship between the different adenosine antagonists and the patterns of activity that they elicited.

**FIG. 1. Effects of adenosine antagonists on repetitive bursting in CA3.**

A: typical pattern of bursting observed after superfusion with the nonselective adenosine receptor antagonist 8-\( p \)-sulfophenyltheophylline (8SPT; bursting was not observed in this slice before 8SPT superfusion). In this and all subsequent figures, \( l \), reciprocal of the interburst interval (instantaneous burst frequency) indicated at the time that the burst occurred. After an initial peak in the burst rate, it remained relatively stable for the duration of 8SPT superfusion (horizontal line), and throughout the following 60-min washout period. B: individual examples of bursts that occurred at the time points indicated in A. C: similar pattern of bursting occurring with superfusion with the A1 selective antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX; horizontal line; washout not shown). Note the significantly longer latency to the onset of bursting in DPCPX. D: examples of individual bursts that occurred at the time points indicated in C.
adenosine, which inhibited responses by 

Wang 1986; Dunwiddie 1980). In a group of four slices su-

bursting was initiated by 8SPT followed by washout, at which point slices then were superfused with adenosine, which has been shown to block spiking induced by other agents (Ault and Wang 1986; Dunwiddie 1980). In a group of four slices su-

alternative possibility was that the repetitive bursting was simply due to the persistence of the antagonist in the tissue. An

burst rate during the 5 min immediately before the washout period (i.e., from 25 to 30 min of 8SPT superfusion). A second group of slices was treated in an identical fashion, except that they were continuously superfused with 8SPT throughout the experiment (○; n = 4). The mean normalized burst rates at the end of the experiment were 0.47 ± 0.078 for the washout group and 0.49 ± 0.10 for the continuous 8SPT group (not significantly different). A third group of slices (○; n = 7) was treated as illustrated in Fig. 3A. For this group of slices, each point represents the mean normalized evoked fEPSP amplitude during 8SPT washout (adenosine was superfused throughout the experiment at 20 

perfused with 100 µM 8SPT, 40 µM adenosine was added for 15 min after 30 min of 8SPT washout. Adenosine almost immediately terminated bursting (mean latency 1.3 min; Fig. 5A), but the bursting resumed shortly after adenosine washout (mean latency 2.6 min), and the rate and pattern of the bursts were almost identical to what had been observed prior to adenosine superfusion (Fig. 5). Thus inhibition of the bursting per se only transiently affected this pattern of activity.

A recent study has demonstrated that repetitive bursting in the CA3 region leads to long-term potentiation (LTP)-like potentiation of feed-forward excitatory synaptic connections in this area (Bains et al. 1999). Thus we examined the hypothesis that an NMDA receptor-dependent form of plasticity was responsible for induction of the persistent bursting. In initial experiments, we characterized the effect of the competitive NMDA receptor antagonist AP5, and the noncompetitive antagonist dizocilpine, on 8SPT-induced bursting. The antagonists were applied by superfusion beginning 10 min (AP5) or 20 min (dizocilpine) before 8SPT superfusion. The presence of NMDA antagonists during 8SPT superfusion did not affect the latency to onset of bursting, which if anything was reduced slightly when compared with slices superfused with 100 µM 8SPT alone (Table 3). The antagonists typically did not affect the initial pattern of bursting (Fig. 6), but there was a modest reduction in the rate of bursting that was observed after 30 min of superfusion with the NMDA antagonists + 8SPT (Table 3), which was statistically significant for the 25 µM AP5 group (P < 0.05; Table 3). The reduction in rate was due at least in part to the fact that in several slices, bursting began shortly after the onset of 8SPT superfusion but was not maintained for

![FIG. 4. Time course of 8SPT washout. Mean normalized burst rate is shown for a group of slices superfused for 30 min with 8SPT and then superfused with control artificial cerebrospinal fluid (ACSF) for 60 min (○; n = 6). The period of drug superfusion is not shown, and the washout period began at time 0. Rate of bursting for each slice was normalized to the mean burst rate during the 5 min immediately before the washout period (i.e., from 25 to 30 min of 8SPT superfusion). A second group of slices was treated in an identical fashion, except that they were continuously superfused with 8SPT throughout the experiment (○; n = 4). The mean normalized burst rates at the end of the experiment were 0.47 ± 0.078 for the washout group and 0.49 ± 0.10 for the continuous 8SPT group (not significantly different). A third group of slices (○; n = 7) was treated as illustrated in Fig. 3A. For this group of slices, each point represents the mean normalized evoked fEPSP amplitude during 8SPT washout (adenosine was superfused throughout the experiment at 20 µM); washout of 8SPT began at time 0. fEPSP responses were normalized such that 0 corresponded to the response amplitude in 20 µM adenosine (period B in Fig. 3), and 1 corresponded to the mean amplitude during the period from 25 to 30 min of 8SPT superfusion (C in Fig. 3). Some of the points are offset slightly for clarity.](http://jn.physiology.org/)

![FIG. 3. Characterization of 8SPT washout. To determine the rate at which 8SPT washed out of slices, slices initially were superfused with 20 µM adenosine, which inhibited responses by ~75% (B), and then 8SPT (100 µM) was added to the buffer for 30 min. This concentration of 8SPT completely reversed the effect of adenosine, as well as the effects of endogenous adeno-

sine, resulting in a response amplitude significantly larger than the initial control amplitude (C). This is consistent with a reversal of the effects of the endogenous adenosine that normally inhibits transmission at these synapses under control conditions (Dunwiddie and Diao 1994; Dunwiddie and Hoffer 1980). During 60 min of subsequent 8SPT washout, the response amplitude returned to the original adenosine-inhibited baseline as the antagonist was washed from the tissue (D). Finally, on washout of adenosine the response amplitude increased to near the original baseline (E). Normalized mean ± SE amplitude of the field excitatory postsynaptic potential (fEPSP) response during the time periods denoted by lettered bars in A are shown in B for a group of 7 slices treated with an identical protocol to that shown in A. Detailed time course of the 8SPT washout for this group of slices is illustrated in Fig. 4.](http://jn.physiology.org/)
the full 30 min of antagonist superfusion (e.g., Fig. 6B). Nevertheless, the most apparent effect of the NMDA receptor antagonists was that they completely blocked the persistent spiking after washout of 8SPT in every slice tested. This was observed in 6/6 slices treated with 25 \( \mu \text{M} \) AP5, 4/4 slices with 50 \( \mu \text{M} \) AP5, and 4/4 slices with 10 \( \mu \text{M} \) dizocilpine. This was not simply due to a prolonged reduction in excitability after AP5 treatment because when two slices were treated for a second time with 8SPT after washout of 50 \( \mu \text{M} \) AP5, both showed normal bursting activity (Fig. 6A). It was also apparent that once the persistent spiking was initiated, it was no longer inhibited by AP5; in 4/4 slices treated with 50 \( \mu \text{M} \) AP5 after washout of 8SPT, there was no effect on the frequency of the bursting (Fig. 6C). Thus NMDA receptor antagonists blocked the establishment of persistent spiking activity, but only if they were present during the 8SPT superfusion.

The observation that NMDA receptor antagonists reduced the frequency of bursting even while the 8SPT was present suggested that they could be blocking the persistent effect by inhibiting the magnitude and/or frequency of bursts, which, in turn, reduced the probability of inducing the long-term plasticity through a non-NMDA receptor-dependent mechanism. To rule out this possibility, we examined the effects of a protein kinase inhibitor that has been reported to block NMDA receptor-dependent LTP in the CA1 region but that does not appear to interact directly with NMDA receptors. KN-62 is a selective inhibitor of rat brain Ca\(^{2+}/\) calmodulin-dependent protein kinase II that acts by binding directly to the calmodulin site of the enzyme (Tokumitsu et al. 1990). Previous studies have shown that KN-62 blocks NMDA receptor-dependent forms of LTP (e.g., in the Schaffer collateral/commissural inputs to the CA1 region) but

![FIG. 5. Adenosine superfusion only transiently inhibits persistent bursting. This slice was superfused with adenosine (40 \( \mu \text{M} \)) after 30 min of washout of 8SPT. A 15-min period of adenosine superfusion completely inhibited the bursting, but it began again almost immediately on subsequent washout of the adenosine. Frequency of the bursting (A) and the amplitude and duration of the bursts (B) were essentially identical before and after interruption of the bursting with adenosine. Similar effects of adenosine superfusion were observed in 4 slices.](http://www.jn.physiology.org/doi/fig/10.1152/jn.01037.2001)

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<th>Treatment</th>
<th>Latency to Onset, min</th>
<th>Frequency at 30 min, Hz</th>
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<tr>
<td>8SPT (100 ( \mu \text{M} ))</td>
<td>3.3 ± 0.21 (25)</td>
<td>0.19 ± 0.013 (24)</td>
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<tr>
<td>8SPT (100 ( \mu \text{M} )) + AP5 (25 ( \mu \text{M} ))</td>
<td>2.78 ± 0.14 (10)</td>
<td>0.14 ± 0.03* (7)</td>
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<tr>
<td>8SPT (100 ( \mu \text{M} )) + AP5 (50 ( \mu \text{M} ))</td>
<td>2.43 ± 0.24 (5)</td>
<td>0.14 ± 0.06 (5)</td>
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<tr>
<td>8SPT (100 ( \mu \text{M} )) + dizocilpine (10 ( \mu \text{M} ))</td>
<td>2.9 ± 0.42 (6)</td>
<td>0.17 ± 0.10 (4)</td>
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<td>8SPT (100 ( \mu \text{M} )) + KN-62 (3 ( \mu \text{M} ))</td>
<td>3.14 ± 0.72 (6)</td>
<td>0.23 ± 0.03 (6)</td>
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* \( P < 0.05 \) versus 8SPT alone.
does not interfere with NMDA receptor-independent LTP (Bortolotto and Collingridge 1998; Ito et al. 1991). Slices were superfused with 3 μM KN-62 for 20 min before the treatment with 8SPT (100 μM). Neither the latency to onset of bursting (3.1 ± 0.72 min) nor the frequency of bursting after 30 min (0.23 ± 0.03 Hz) was significantly different from what was observed after 8SPT alone. However, 4/6 slices stopped bursting during the 60-min washout period, and the decline in the mean rate of spiking was virtually identical to the time course for 8SPT washout as determined by the preceding experiments with the fEPSP responses. The mean normalized frequency of spiking in the KN-62 slices after 20 min of 8SPT washout (0.74 ± 0.16, n = 6) was not significantly different from the 8SPT control slices, but by 40 min was decreased significantly compared with controls (0.21 ± 0.11; P < 0.02) and at 60 min had essentially reached baseline (0.06 ± 0.04; P < 0.001 vs. 8SPT washout). Thus the NMDA receptor antagonists AP5 and dizocilpine, as well as the Ca\(^{2+}\)/calmodulin-dependent protein kinase II inhibitor KN-62, significantly inhibited the persistent spiking induced by 8SPT.

**DISCUSSION**

The results of the present experiments confirm the basic observations made in most previous studies concerning the persistent effects of adenosine receptor antagonists on repetitive bursting in the CA3 region. We observed that after superfusion with adenosine antagonists, repetitive bursting occurs that persists well beyond the termination of superfusion with the antagonist ligand. However, these experiments also have identified a likely cellular mechanism that is responsible for the persistence of this bursting. Our results demonstrate that the persistence of the bursting reflects the involvement of an LTP-like process, which is dependent both on activation of NMDA receptors and the activity of a Ca\(^{2+}\)/calmodulin-dependent protein kinase. Bains et al. (1999) showed that the repetitive firing of CA3 pyramidal neurons during bursts induced by high K\(^+\) potentiates the recurrent excitatory connections between CA3 neurons and proposed that LTP of these synapses may alter the functional circuitry of this region in such a way that bursting persists independently of any subsequent experimental interventions. It appears likely that adenosine receptor antagonists initiate a similar process, which then continues independently of occupation of adenosine receptors by the antagonist. Thus what makes the bursting self-sustaining is not the activity per se, (i.e., recurrent excitatory activity in the slice), but changes in the strength of intrinsic hippocampal synapses that increase the excitability of the CA3 network to the point where periodic bursting occurs. This hypothesis is supported by the observation that once persistent bursting was initiated, complete inhibition of such activity for 10–15 min with adenosine (Fig. 5) only transiently suppressed the bursting, which recurred immediately upon washout of the adenosine.

In terms of some of the alternative mechanisms that have been proposed to account for persistent bursting, the present experiments provide evidence to resolve what have been somewhat controversial issues. A trivial explanation for the persistence of the bursting, viz., that it reflects the persistence of the antagonist in the tissue, can be ruled out as a basis for the persistent effects of 8SPT. We found that normal responsiveness of A1 receptors to adenosine is reinstated within an hour after washout of 8SPT (Fig. 3), indicating that the antagonist no longer occupies the receptors at a time when bursting is still occurring. However, as has been correctly pointed out by Chesi and Stone (1997), many adenosine receptor antagonists, particularly the more potent ones such as DPCPX, are highly lipophilic compounds that partition into membranes and as such are very slow to wash out of slices. Antagonists such as DPCPX dissociate rapidly (within minutes) from A1 receptors, which can be demonstrated by the rapid onset of adenosine-mediated inhibition induced by adding high concentrations of adenosine in the presence of DPCPX (Dunwiddie, unpublished data). The limiting factor in the termination of the effects of...
antagonists such as DPCPX in slices (which can be >1 h in some cases) appears to be the rate at which they leave sites in the membrane and not the rate of dissociation from the receptors. Thus persistent bursting induced by agents such as DPCPX could reflect insufficient washout of the antagonist, a persistent underlying process, or in most cases, both.

Because persistent bursting has been observed consistently with A1-selective antagonists such as DPCPX (Alzheimer et al. 1989; Chesi and Stone 1997), whereas the persistent effect is only sometimes observed with nonselective antagonists such as theophylline (persistent, Ault et al. 1987; transient, Ault and Wang 1986; Chesi and Stone 1997), it has been suggested that the effects of A1-selective antagonists are different from nonselective antagonists. However, the results of the present study do not support this distinction, in that there was no difference in the incidence of bursting between highly selective antagonists such as DPCPX, and a relatively nonselective antagonist (8SPT) used in a concentration where both A1 and A2 receptors would have been blocked.

The results of the present study suggest that an LTP-like phenomenon supports the persistent component of bursting induced by adenosine receptor antagonists. As with NMDA receptor-dependent LTP, persistent bursting could be blocked by the presence of both competitive (AP5) and noncompetitive (dizocilpine) NMDA receptor antagonists when they were present during the initial period of bursting, but once persistent bursting was initiated, it was no longer sensitive to the antagonists. In this respect, persistent bursting induced by adenosine antagonists is similar to both LTP and persistent bursting elicited by other agents that do not interact with adenosine receptors (Bains et al. 1999; Schneiderman et al. 1994). As has been noted previously (Schneiderman et al. 1994), the epileptiform bursts appear to be at least partially supported by NMDA receptor-mediated transmission because NMDA antagonists reduce the amplitude, duration, and frequency of bursting (Bains et al. 1999). Conversely, it has been reported that superfusion with 0 Mg2+ buffer (which would enhance NMDA responses), increases or in some cases initiates bursting (Chesi and Stone 1997), which is consistent with this hypothesis. For these reasons, it was particularly important to demonstrate that persistent bursting induced by 8SPT also could be blocked by other agents that block LTP but do not interact with adenosine receptors. Thus the inhibition of persistent bursting by KN-62, an inhibitor of Ca2+/calmodulin-dependent protein kinase, confirms that the cellular mechanisms underlying the two effects is similar and suggests that the effects of the NMDA receptor antagonists are not simply due to an inhibition of the bursting below some threshold that is required to induce the persistent effect. These results stand in contrast to the findings of Alzheimer et al. (1993), who reported that NMDA antagonists did not reverse the persistent effects of DPCPX. However, in their experiments with NMDA antagonists, it was unclear whether DPCPX was completely washed out of the tissue. Thus the persistence of bursting in the presence of NMDA antagonists in their experiments may have resulted from the continued occupancy of adenosine receptors by DPCPX. One observation that cannot be explained by an LTP-like mechanism is the persistent depolarizing effect of DPCPX on CA3 neurons (Alzheimer et al. 1993). This study reported that superfusion with both DPCPX as well as adenosine deaminase produced persistent decreases in the membrane potential that were also very long-lasting. These results suggest that there may be other enduring consequences of reversing the effects of endogenous adenosine that may not depend on LTP-like mechanisms and will require further investigation.

A final issue of concern relates to the functional significance of these observations in relation to the effects of caffeine, theophylline, and other adenosine receptor antagonists in man. Although the present results suggest that there could be adverse long-term consequences to the use of these pharmacological agents, clinical observations on individuals using these drugs generally does not support this idea. Seizures induced by theophylline do occur, but usually only in the case of overdose, and they appear to involve other mechanisms in addition to the antagonism of adenosine receptors. In this respect, it should be pointed out that “normal” hippocampal slices probably correspond to a proconvulsant state, in that some of the recurrent inhibitory pathways that normally suppress bursting in vivo are truncated in the slice cutting procedure. In addition, most studies of bursting in hippocampal slices use media with reduced Ca2+ and Mg2+ concentrations, and in some cases elevated K+, to observe reliable bursting, and these conditions are not normally found in vivo. Thus it is unlikely that concentrations of caffeine and theophylline within the normal ranges induce hippocampal bursting in intact animals or humans, and correspondingly, it is unlikely that they would induce any persistent effects. However, in individuals with seizure disorders, it is quite possible that the excitatory actions of adenosine receptor antagonists could initiate bursting, which then might induce persistent effects via an NMDA receptor-dependent mechanism. For this reason, the use of adenosine antagonists in such individuals would be contraindicated.

In conclusion, the present studies have demonstrated that superfusion of hippocampal brain slices with adenosine receptor antagonists induces epileptiform bursting, and that after drug washout, this bursting continues well beyond the duration of antagonist occupation of adenosine receptors. This effect is induced by the displacement of endogenous adenosine from adenosine A1 receptors, but simultaneous occupation of A2A or A2B receptors by an antagonist does not modify the incidence of the persistent response. The induction of this persistent bursting requires activation of NMDA receptors, as well as Ca2+/calmodulin-dependent protein kinase. These results suggest that under certain conditions, the use of methylxanthines could lead to persistent increases in brain excitability.

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