

## DISINHIBITION OF HIPPOCAMPAL CA3 NEURONS INDUCED BY SUPPRESSION OF AN ADENOSINE A<sub>1</sub> RECEPTOR-MEDIATED INHIBITORY TONUS: PRE- AND POSTSYNAPTIC COMPONENTS

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**Abstract**—Intracellular recordings were performed on hippocampal CA3 neurons *in vitro* to investigate the inhibitory tonus generated by endogenously produced adenosine in this brain region. Bath application of the highly selective adenosine A<sub>1</sub> receptor antagonist 1,3-dipropyl-8-cyclopentylxanthine at concentrations up to 100 nM induced both spontaneous and stimulus-evoked epileptiform burst discharges. Once induced, the 1,3-dipropyl-8-cyclopentylxanthine-evoked epileptiform activity was apparently irreversible even after prolonged superfusion with drug-free solution. The blockade of glutamatergic excitatory synaptic transmission by preincubation of the slices with the amino-3-hydroxy-5-methyl-4-isoxazolpropionic acid receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (10  $\mu$ M), but not with the *N*-methyl-D-aspartate receptor antagonist D-2-amino-5-phosphonovaleric acid (50  $\mu$ M), prevented the induction of epileptiform activity by 1,3-dipropyl-8-cyclopentylxanthine. The generation of the burst discharges was independent of the membrane potential, and the amplitude of the slow component of the paroxysmal depolarization shift increased with hyperpolarization, indicating that the 1,3-dipropyl-8-cyclopentylxanthine-induced bursts were synaptically mediated events. Recordings from tetrodotoxin-treated CA3 neurons revealed a strong postsynaptic component of endogenous adenosinergic inhibition. Both 1,3-dipropyl-8-cyclopentylxanthine and the adenosine-degrading enzyme adenosine deaminase produced an apparently irreversible depolarization of the membrane potential by about 20 mV. Sometimes, this depolarization attained the threshold for the generation of putative calcium spikes, but no potential changes resembling paroxysmal depolarization shift-like events were observed.

At the concentrations used in electrophysiological experiments (30–100 nM), 1,3-dipropyl-8-cyclopentylxanthine displayed only a negligible inhibitory action on total cyclic nucleotide phosphodiesterase activity measured by means of a radiochemical assay in a homogenate of the rat cerebral cortex. Furthermore, even high concentrations of the selective phosphodiesterase inhibitor rolipram (10  $\mu$ M), which displays no affinity to adenosine receptors, did not mimic the electrophysiological actions of 1,3-dipropyl-8-cyclopentylxanthine, thus excluding the possibility that the effects of the A<sub>1</sub> receptor antagonist on neuronal discharge behavior can be ascribed to an inhibition of phosphodiesterases.

The present data demonstrate that endogenously released adenosine exerts a vigorous control on the excitability of hippocampal CA3 neurons on both the pre- and postsynaptic sites. The long-lasting disinhibition following a transient suppression of adenosinergic inhibition strongly suggests that, besides its well-known short-term effects on neuronal activity, adenosine might also contribute to the long-term control of hippocampal excitability.

It is now generally accepted that a significant amount of endogenous adenosine is permanently present in the extracellular space of the mammalian CNS.<sup>17–19,36,43,44,48</sup> Measurements *in vivo* and *in vitro* revealed basal levels of adenosine in the cerebrospinal fluid ranging from 1 to 5  $\mu$ M.<sup>22,56</sup> At these concentrations, adenosine is capable of reducing transmitter release<sup>29</sup> and decreasing postsynaptic excitability.<sup>23,24,26</sup> Electrophysiological studies have

shown that adenosine exerts a potent short-term control on hippocampal excitability. Rises in extracellular adenosine produce a rapid depression of neuronal activity that is quickly reversible when adenosine levels return to baseline.<sup>1,2,23,24,48</sup> While the immediate actions of adenosine are well established, some preliminary data from our and other laboratories suggest that adenosine might also contribute to the long-term control of neuronal excitability.<sup>3,4,6,51</sup> In these studies, both a transient increase in extracellular adenosine as well as a transient removal of the adenosine tonus have been tentatively linked to long-lasting changes of hippocampal responsiveness. The present study aims to elaborate on this putative new role for adenosine.

Adenosine exerts its actions on CNS neurons via two receptor subtypes, the A<sub>1</sub> and A<sub>2</sub> receptors.<sup>32,55</sup> The adenosine receptors can be blocked by xanthines such as caffeine and theophylline. Therefore, it has

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**Abbreviations:** ACSF, artificial cerebrospinal fluid; ADA, adenosine deaminase; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; D-APV, D-2-amino-5-phosphonovaleric acid; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; EDTA, ethylenediaminetetra-acetate; EPSP, excitatory postsynaptic potential; fEPSP, extracellularly recorded field EPSP; IBMX, 3-isobutyl-1-methylxanthine; NMDA, *N*-methyl-D-aspartate; PDS, paroxysmal depolarization shift; TTX, tetrodotoxin.

been suggested that the well-known excitatory effects of these xanthines observed both *in vivo*<sup>49</sup> and *in vitro*<sup>8,20,25,38</sup> are due to their antagonistic action at adenosine receptors. However, these xanthine derivatives do not discriminate between A<sub>1</sub> and A<sub>2</sub> receptors.<sup>16</sup> In addition, they also interfere with mechanisms not related to adenosinergic actions, e.g. inhibition of phosphodiesterases.<sup>10</sup> Consequently, these antagonists cannot be used as selective tools to investigate the extent and mechanisms of the endogenous adenosinergic tonus.

The development of a highly selective adenosine A<sub>1</sub> receptor antagonist, 1,3-dipropyl-8-cyclopentylxanthine (DPCPX),<sup>14,34</sup> provided the possibility to study the actions produced by endogenously released adenosine via adenosine A<sub>1</sub> receptors. Using DPCPX, we have recently demonstrated that, in hippocampal slices, the excitability of area CA3 neurons is controlled by an A<sub>1</sub> receptor-mediated adenosine tonus which appeared to be equally potent to that exerted by GABA via the GABA<sub>A</sub> receptor.<sup>4</sup> Most intriguingly, a transient application of DPCPX was sufficient to cause sustained epileptiform activity. Since a concomitant action of DPCPX at the same adenosine receptor, namely the suppression of an adenosine-evoked K-current, was completely reversible in the same slices that displayed sustained epileptiform activity,<sup>4</sup> the DPCPX-induced long-term changes in excitability cannot be ascribed to an irreversible blockade by DPCPX of A<sub>1</sub> receptors or a persistence of the drug in the tissue. This notion was supported by a recent quantitative pharmacological study showing that the actions of DPCPX in hippocampus are due to a competitive inhibition of A<sub>1</sub> receptors, the apparent dissociation constant ( $K_D$ ) being 3.3 nM.<sup>2</sup>

The role of presynaptically vs postsynaptically located A<sub>1</sub> receptors in the modulation of hippocampal excitability is still controversial. For example, it has been reported that the non-selective adenosine receptor antagonist caffeine induces epileptiform discharges in guinea-pig hippocampal CA3 neurons solely by postsynaptic mechanisms.<sup>38</sup> On the other hand, intracellular recordings from neurons in an organotypic hippocampal culture provided evidence that adenosine antagonists enhance the presynaptic release of glutamate, leading to an increase in frequency and size of spontaneous excitatory postsynaptic potentials (EPSPs).<sup>40</sup> Furthermore, these antagonists facilitate the potassium-stimulated and basal release of glutamate from cerebellar granule cells in culture.<sup>40</sup> The present study was designed to assess the contribution of pre- and/or postsynaptic elements to the generation of DPCPX-induced epileptiform activity.

As a xanthine derivative, DPCPX may display some inhibitory action on brain phosphodiesterases.<sup>45,46</sup> Since inhibition of cAMP phosphodiesterases can enhance hippocampal excitability and sometimes, especially upon application of 3-isobutyl-1-methylxanthine (IBMX), induce epileptiform ac-

tivity,<sup>50</sup> we determined the actions of DPCPX on total phosphodiesterase activity in a homogenate from rat cerebral cortex by means of a radiochemical assay. To our knowledge, there is no evidence for a differential distribution of brain phosphodiesterase isozymes between cortex and hippocampus. We also compared the effects of the selective cAMP phosphodiesterase inhibitor rolipram<sup>10</sup> on evoked field potentials recorded extracellularly in stratum radiatum of area CA3 to those induced by DPCPX.

## EXPERIMENTAL PROCEDURES

### *Preparation and maintenance of slices*

Experiments were performed on slices obtained from the guinea-pig hippocampus. The procedure of slice preparation was essentially the same as described previously.<sup>1-4</sup> In brief, the animals (180–300 g; source: Savo, D-88350 Kieslegg, Germany) were anesthetized with ether and decapitated. Following removal of the brain, the hippocampi were dissected and cut into two halves. Transverse slices of a nominal thickness of 500  $\mu$ m were prepared by means of a Vibratome and stored in a holding chamber at room temperature for at least 90 min. Then, single slices were transferred to the recording chamber, where they were fixed between two nylon meshes. The slices were kept submerged in artificial cerebrospinal fluid (ACSF) consisting of (in mM): NaCl 125, KCl 3, CaCl<sub>2</sub> 2, MgCl<sub>2</sub> 1.3, NaHCO<sub>3</sub> 25, NaH<sub>2</sub>PO<sub>4</sub> 1.25 and D-glucose 10. The solution was continuously gassed with a mixture of 95% O<sub>2</sub> and 5% CO<sub>2</sub> in order to obtain a pH value of 7.4 at a recording temperature of 31–32°C.

### *Electrophysiology*

Intracellular recordings from CA3 pyramidal neurons were performed using glass microelectrodes filled with 3 M KCl. The resistance of these electrodes ranged between 30 and 80 M $\Omega$ . Evoked field potentials were recorded from stratum radiatum of area CA3 by means of microelectrodes filled with 3 M NaCl (2–4 M $\Omega$ ). All signals were recorded and amplified using an npi SEC 1L amplifier (npi, Tamm, Germany). For voltage recordings and current injection, the amplifier was operated in the active bridge mode. Throughout the experiment, membrane potential was monitored on a chart recorder (Gould System Instruments, Cleveland, OH, U.S.A.) and stored on magnetic tape (TEAC MR30, Tokyo, Japan, frequency response d.c. – 2.5 kHz). To monitor the cells' input resistances, hyperpolarizing current pulses (0.2 nA, 500 ms) were injected at a frequency of 0.1–0.2 Hz. Synaptic potentials were elicited by means of a concentric bipolar electrode positioned in the mossy fiber pathway. Stimulation was performed at a frequency of 0.05 Hz, and the duration of the stimulus was 50  $\mu$ s. Neurons were selected on the basis of their resting membrane potentials (> –60 mV), their input resistances (> 70 M $\Omega$ ) and their spike overshoots (> 15 mV). Intra- and extracellular signals were digitized (sampling rate  $\geq$  2 kHz) by means of a laboratory computer system (CED 1401, Cambridge, U.K. in conjunction with a PC AT) and stored for off-line analysis. Field potentials were averaged on-line (four to five sweeps).

### *Application of drugs*

Drugs used in electrophysiological experiments were tetrodotoxin (TTX; Serva, Heidelberg, Germany), DPCPX (RBI, Natick, U.S.A.), adenosine deaminase (ADA; Boehringer Mannheim, Germany), 4-(3-cyclopentylloxy-4-methoxy-phenyl)-2-pyrrolidone (rolipram; Schering, Berlin, Germany), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and D-2-amino-5-phosphonovalerate (D-APV; both from

Tocris Neuramin, Buckhurst Hill, U.K.). The xanthines, rolipram and CNQX were taken from stock solutions (10 mM, dissolved in dimethylsulfoxide) and added to the ACSF to reach the final concentrations desired. The maximum solvent concentration never exceeded 0.1%. At this concentration, dimethylsulfoxide neither affected the electrophysiological properties of CA3 neurons nor the amplitude and rise time of the extracellularly recorded field EPSP (fEPSP). All drugs were applied by addition to the ACSF, which was continuously circulated at a flow rate of 3–5 ml/min.

#### Measurement of phosphodiesterase activity

Rat cerebral cortices were isolated and homogenized in 10 volumes of buffer containing (in mM): BisTris 20, dithiothreitol 5, phenylmethanesulfonylfluoride 0.05, benzamidine 2, EDTA 2, sodium acetate 50, pH 6.5. Genapol (0.15%, v/v) was included to facilitate extraction of membrane bound phosphodiesterases.<sup>39</sup> The homogenate was sonicated four times for 20 s at 65 W and then centrifuged for 20 min at 39,000 *g* and 4°C. The supernatants were diluted with buffer consisting of 20 mM BisTris, 5 mM dithiothreitol and 50 mM sodium acetate (pH 6.5) to reach final protein concentrations of 0.4–0.5 mg/ml.

Phosphodiesterase activity was assayed according to a radiochemical method initially described by Thompson *et al.*<sup>53</sup> and modified by Bauer and Schwabe.<sup>9</sup> In a first step, <sup>3</sup>H-labeled adenosine-3',5'-monophosphate (<sup>3</sup>H]cAMP) was hydrolysed to [<sup>3</sup>H]5'-adenosine monophosphate (<sup>3</sup>H]5'-AMP). In the second step, [<sup>3</sup>H]5'-AMP was degraded to [<sup>3</sup>H]adenosine by snake venom 5'-nucleotidase. Then, the product was separated from unconverted [<sup>3</sup>H]cAMP using anion-exchange chromatography.

The incubation medium contained 50 mM Tris-HCl (pH 8.0), 10 mM MgCl<sub>2</sub>, 4 mM 2-mercaptoethanol, 0.125 mg/ml bovine serum albumin, 100,000 c.p.m. pre-purified [<sup>3</sup>H]cAMP (Amersham-Buchler, Braunschweig, Germany; 20–30 Ci/mmol) and 1  $\mu$ M or 100  $\mu$ M cAMP. After a 3 min preincubation at 30°C, the reaction was started by addition of 100  $\mu$ l of homogenate. The incubation was performed at 30°C for 10 min and the reaction was stopped by transferring the assay tubes to a water bath at 90°C. After re-equilibration to room temperature, an excess of 5'-nucleotidase (snake venom from *Ophiophagus hannah*, 1 mg/ml) was added to each tube and the samples were incubated again at 30°C for 10 min. This reaction was terminated by applying an aliquot of 0.2 ml to a QAE A-25 Sephadex column (7  $\times$  15 mm in Pasteur pipettes) previously equilibrated with 3 ml of 30 mM ammonium formate (adjusted to pH 6 with formic acid). The eluate was collected directly into scintillation vials. The columns were washed with 2 ml ammonium formate buffer and these eluates were collected into the same vials. Radioactivity was measured by determination of c.p.m. in 15 ml of a scintillation fluid for aqueous samples ("Ready valuc", Beckman, Munich, Germany). Assay blanks were prepared with either no or heat-inactivated homogenate. Recovery was determined from samples containing 10  $\mu$ g of commercial phosphodiesterase (Boehringer Mannheim, Germany). The xanthine derivatives and rolipram were dissolved in dimethylsulfoxide (stock solutions of 10–100 mM) and added to the incubation medium to give final concentrations between 0.01 and 300  $\mu$ M. Even at the highest concentrations achieved (0.3%), the solvent had no effect on phosphodiesterase activity.

Protein was determined using the bicinchoninic acid assay procedure described by Smith *et al.*<sup>47</sup> The inhibitory efficiency of xanthines and rolipram on phosphodiesterase activity is expressed as percent inhibition of total activity (pmol/ml/min), which was determined in the absence of these drugs. All assays were performed in triplicate and each experiment was repeated at least three times in different animals.

All values are given as mean  $\pm$  S. D. Statistical comparisons of mean values were made using the Student *t*-test.

## RESULTS

### Induction of epileptiform activity by 1,3-dipropyl-8-cyclopentylxanthine

In agreement with previous accounts,<sup>28</sup> the intrinsic firing pattern of CA3 neurons observed in normal ACSF was found to be strongly voltage-dependent, consisting of either single spike activity at more depolarized potentials or burst discharges of two to four action potentials riding on top of a small depolarizing envelope at more negative potentials. As indicated by the high frequency of spontaneously occurring postsynaptic potentials, most CA3 neurons almost continuously received synaptic input.

Within 15–20 min of bath application of 30–100 nM DPCPX, a complete change in neuronal discharge behavior was observed (Fig. 1A). The normal firing pattern was converted to epileptiform activity with rhythmically occurring paroxysmal depolarization shifts (PDSs) generating trains of action potentials. Similarly, synaptic stimulation which, under control conditions, evoked a single action potential, now triggered the discharge of a PDS-like event (Fig. 1B). The disinhibitory action of DPCPX (30–100 nM) was a consistent finding in all CA3 neurons tested. In 20 out of 22 cells, DPCPX induced epileptiform burst discharges. In the remaining two neurons, application of DPCPX led to a depolarization of the membrane potential by 3 and 6 mV, respectively, associated with an increase in input resistance and an enhanced firing rate. Once initiated, the DPCPX-induced epileptiform activity never ceased, even after prolonged (> 3 h) superfusion with normal ACSF (Fig. 1). To confirm that the epileptiform activity was generated within the neuronal network of area CA3, i.e. independent of external inputs from other parts of the hippocampus, experiments were performed using so-called "CA3 mini-slices", from which all other hippocampal regions had been surgically removed (*n* = 3). When tested under these conditions, DPCPX was still able to produce the typical PDS-like activity (data not shown).

### Contribution of synaptic mechanisms to the generation of 1,3-dipropyl-8-cyclopentylxanthine-induced epileptiform activity

To study whether the DPCPX-induced burst discharges represent synaptically mediated or intrinsically generated events, the epileptogenic action of the xanthine derivative was tested in the presence and absence of intact excitatory synaptic transmission. In these experiments (*n* = 4), EPSPs were blocked by the amino-3-hydroxy-5-methyl-4-isoxazolpropionic acid receptor antagonist CNQX.<sup>5</sup> As illustrated in Fig. 2Ca–c, bath application of CNQX (10  $\mu$ M) almost completely suppressed excitatory synaptic transmission at the mossy fiber–CA3 cell synapse

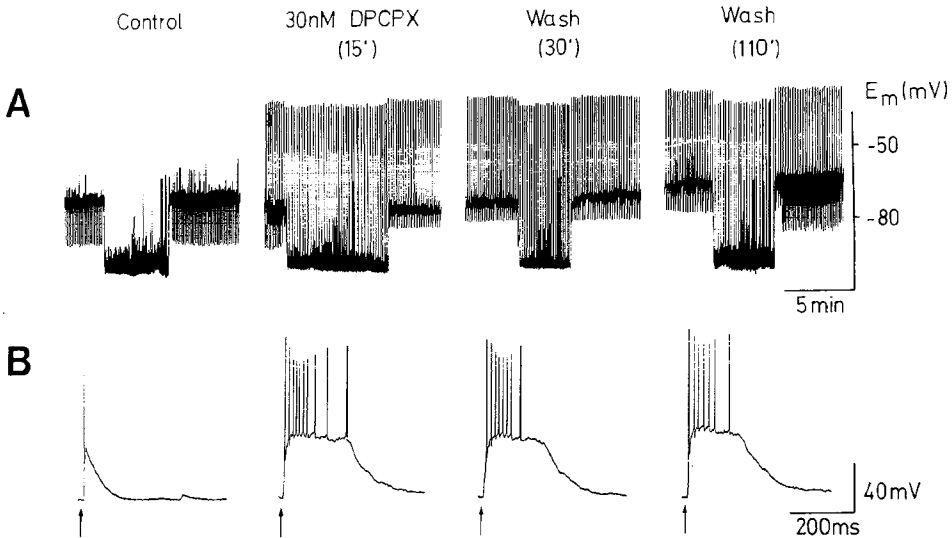


Fig. 1. Effects of the adenosine  $A_1$  receptor antagonist DPCPX on the spontaneous and stimulus-evoked discharge behavior of CA3 pyramidal cells. A shows parts of a chart record of the membrane potential of a CA3 neuron (resting membrane potential:  $-72$  mV; input resistance:  $85$  M $\Omega$ ). The downward voltage deflections were due to hyperpolarizing current pulses ( $0.2$  nA,  $500$  ms) which were injected intracellularly to monitor input resistance. After the onset of the DPCPX-induced burst discharges, the current pulses were only temporarily applied. During electrical stimulation, the membrane potential was hyperpolarized to inhibit single spike discharge. In B, samples of postsynaptic responses are depicted on a faster time scale. The times given in A correspond to the records shown in B. For illustration, stimulus artifacts were removed. The time of stimulation is indicated by arrows. Under control conditions, the stimulus intensity was adjusted to evoke one action potential. This stimulus strength was used throughout the experiment.

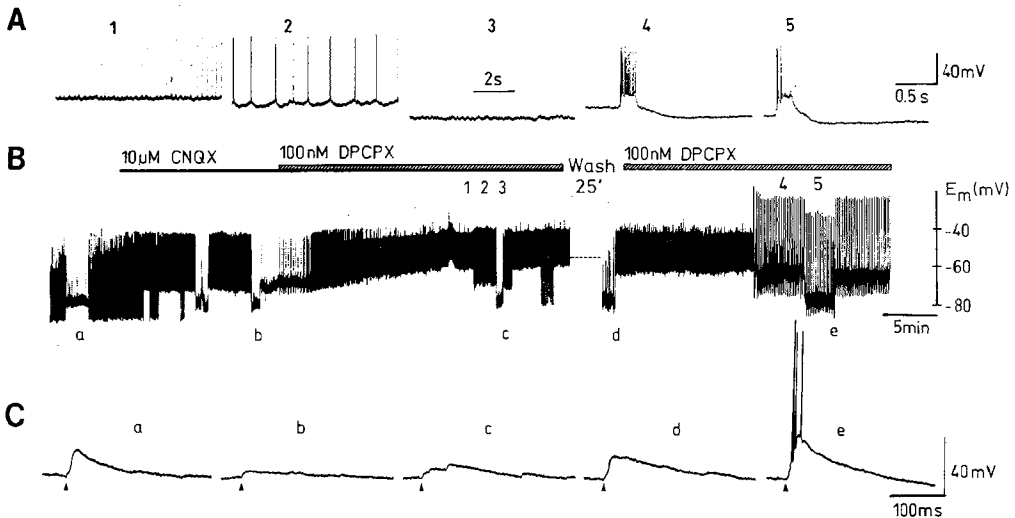


Fig. 2. Suppression of the epileptogenic action of DPCPX by the amino-3-hydroxy-5-methyl-4-isoxazol-propionic acid receptor antagonist CNQX. In B, a chart record of the membrane potential of a CA3 neuron (resting membrane potential:  $-67$  mV; input resistance:  $95$  M $\Omega$ ) is shown. In A and C, samples of both the spontaneous activity (A) and synaptically evoked responses (C) are depicted on faster time scales. The records were taken at the points indicated in B by numbers (A) or letters (C), respectively. Stimulus artifacts were removed and time of stimulation is signified by arrowheads. Postsynaptic potentials (C) were recorded at a membrane potential of  $-80$  mV adjusted by hyperpolarizing current injection. Note the presence of spontaneous, presumably inhibitory, postsynaptic potentials (Cc,d). In A1, the membrane potential was  $-58$  mV. To test the voltage dependence of the spike discharge, the membrane potential was hyperpolarized by current injection to  $-67$  mV (A2) and  $-87$  mV (A3). In contrast to the intrinsically generated spiking activity (A1–A3), the frequency of DPCPX-induced PDS-like discharges was voltage-independent (B4, B5). Note the increase in the amplitude of the slow PDS component upon hyperpolarization to  $-87$  mV (A4 vs A5).

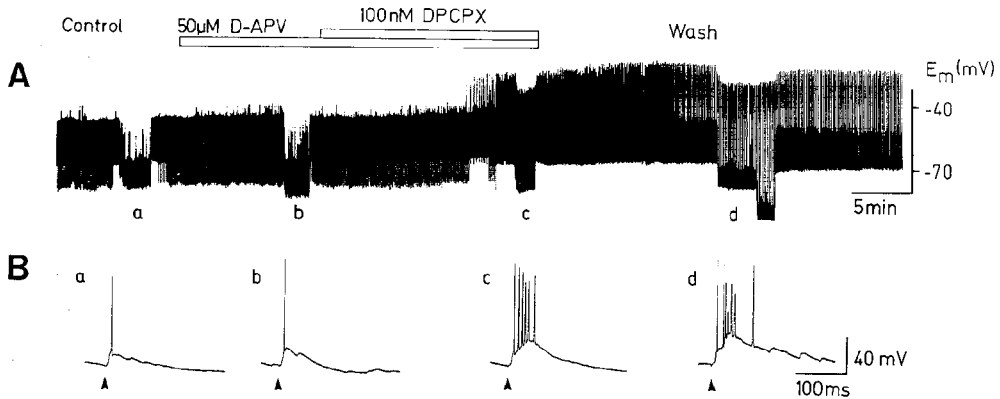


Fig. 3. Failure of the NMDA receptor antagonist D-APV to suppress the epileptogenic action of DPCPX. A displays a continuous chart record of the membrane potential of a CA3 neuron (resting membrane potential:  $-62$  mV; input resistance:  $70$  M $\Omega$ ). Hyperpolarizing current pulses were injected to monitor input resistance. They were switched off upon onset of DPCPX-induced burst discharges. The traces in B illustrate the stimulus-evoked responses on a faster time scale (a–d correspond to the letters in A). The synaptic potentials were recorded at a membrane potential of  $-75$  mV, which was adjusted by hyperpolarizing current injection. Note that, in contrast to control where spikes were suppressed by hyperpolarization (Aa and Ab), an increase in membrane potential did not affect the frequency of burst discharge in the presence of DPCPX (Ad).

without substantially altering resting membrane potential, input resistance or the intrinsic firing pattern of CA3 neurons (Fig. 2B). When added to a CNQX-containing bathing solution, DPCPX ( $100$  nM) was no longer able to induce epileptiform burst discharges. However, the  $A_1$  receptor antagonist still produced a remarkable change in the neurons' intrinsic properties. As shown in Fig. 2B, DPCPX gradually depolarized the membrane potential by about  $10$  mV and substantially increased spontaneous firing. Since the pattern of intrinsic activity of CA3 neurons is known to be strongly voltage-dependent, the firing rate increasing with depolarization and ceasing with hyperpolarization,<sup>28</sup> we adjusted the membrane potential to several holding potentials. As demonstrated by samples 1–3 in Fig. 2A, DPCPX seemed not to alter the typical intrinsic discharge behavior of CA3 neurons. Following washout of CNQX and DPCPX (Fig. 2B, wash 25'), excitatory synaptic transmission fully recovered (Fig. 2Cd), whereas the membrane potential remained depolarized at a stable level (Fig. 2B). After re-establishment of intact excitatory synaptic transmission, reapplication of the same concentration of DPCPX reliably produced spontaneous and evoked epileptiform discharges in all neurons tested (Fig. 2A4, A5, B, Ce). In contrast to the neurons' intrinsic activity, both the rate (Fig. 2Be) and the generation of the PDS-like events were found to be completely independent of the neuronal membrane potential (Fig. 2A4, A5; see also Fig. 3A). Furthermore, the amplitude of the slow depolarization underlying the spike train increased with hyperpolarization (Fig. 2A4, A5), suggesting a synaptic mechanism of generation.<sup>27</sup> As would be expected in this case, the DPCPX-induced epileptiform activity could be reversibly suppressed by CNQX (data not shown).

#### *N-Methyl-D-aspartate independence of 1,3-dipropyl-8-cyclopentylxanthine-induced epileptiform activity*

To find whether induction or maintenance of the sustained epileptiform activity observed in the presence of DPCPX would require the participation of *N*-methyl-D-aspartate (NMDA) receptors, slices were superfused with a bathing solution containing a high concentration of the NMDA receptor antagonist D-APV. When applied in the presence of D-APV ( $50$   $\mu$ M), DPCPX was still capable of inducing epileptiform discharges ( $n = 3$ ). As shown in Fig. 3, both spontaneous and stimulus-evoked bursts were observed following the addition of DPCPX to a D-APV-containing medium. Similar to all other experiments, the disinhibitory action of DPCPX was irreversible within the observation period.

#### *Postsynaptic actions of 1,3-dipropyl-8-cyclopentyl-xanthine*

The DPCPX-induced enhancement of neuronal excitability in the absence of excitatory synaptic transmission indicated a strong postsynaptic component in the action of the  $A_1$  receptor antagonist. To investigate the effects of DPCPX on the postsynaptic membrane independent of spike discharges and synaptic transmission, experiments were performed in the presence of TTX ( $0.6$   $\mu$ M). When tested under these conditions, DPCPX ( $30$ – $100$  nM) induced a pronounced depolarization of the membrane potential, ranging between  $14$  and  $25$  mV ( $19.0 \pm 4.8$  mV,  $n = 6$ ). The onset of the action and the time course of the depolarization were similar to the DPCPX-induced changes observed in TTX-free solution. In three out of six neurons, the membrane potential was depolarized up to the threshold for the generation of TTX-insensitive, putative calcium spikes (Fig. 4A).

Following application of DPCPX, the input resistance first slightly increased then substantially declined as the membrane potential attained more depolarized values (Fig. 4A). This reduction in input resistance was probably due to the strong outward rectification in the depolarizing direction normally observed in these neurons.<sup>1</sup> When kept at control resting membrane potential, the input resistance was found to be slightly enhanced compared to values determined before application of DPCPX (not shown). As in the previous experiments, the DPCPX-induced effects were apparently irreversible, even after prolonged (1–3 h) superfusion with DPCPX-free solution. In the presence of TTX, DPCPX did not produce potential changes resembling PDS-like events.

If the postsynaptic depolarizing action of DPCPX is due to a release from an inhibitory adenosinergic tonus, then enzymatic degradation of endogenous adenosine should mimic the effects of DPCPX on the membrane potential. Therefore, the action of the adenosine degrading enzyme ADA<sup>31</sup> on membrane potential and input resistance was investigated in TTX-treated slices ( $n = 3$ ). Following addition of ADA (10  $\mu\text{g/ml}$ ) to the ACSF, a depolarization by 15–22 mV was observed in all neurons tested (Fig. 4B). Similar to the action of DPCPX, this depolarization was associated with a decrease in input resistance, due to the outward rectifying properties of the cells, and with a low-frequency discharge

of TTX-insensitive action potentials. Furthermore, the effect of the enzyme was apparently irreversible within the observation period.

#### *Effects of 1,3-dipropyl-8-cyclopentylxanthine on phosphodiesterase activity*

To exclude the possibility that the actions of DPCPX described so far were due to the inhibition of cyclic nucleotide phosphodiesterases, we examined the effects of the A<sub>1</sub> receptor antagonist on the total phosphodiesterase activity in a homogenate of the rat cerebral cortex. In the presence of the non-selective phosphodiesterase inhibitor IBMX, a concentration-dependent suppression of total phosphodiesterase activity was observed (Fig. 5A). At high concentrations (300  $\mu\text{M}$ ), IBMX was able to block phosphodiesterase activity by up to 90%. The data could be fitted with a sigmoid function and a half-maximal inhibitory concentration ( $\text{IC}_{50}$ ) was calculated. When 1  $\mu\text{M}$  unlabeled cAMP was included in the assay, the  $\text{IC}_{50}$  for IBMX was found to be 19.4  $\mu\text{M}$ . In the presence of 100  $\mu\text{M}$  cAMP, the  $\text{IC}_{50}$  was 79.8  $\mu\text{M}$ . These values agree with those described in the literature.<sup>10,39</sup> The ability of DPCPX to inhibit cAMP phosphodiesterase activity depended on the substrate concentration present in the assay. At a cAMP concentration of 1  $\mu\text{M}$ , an increasing blockade of phosphodiesterase activity with increasing concentrations of DPCPX was observed (Fig. 5B). However,

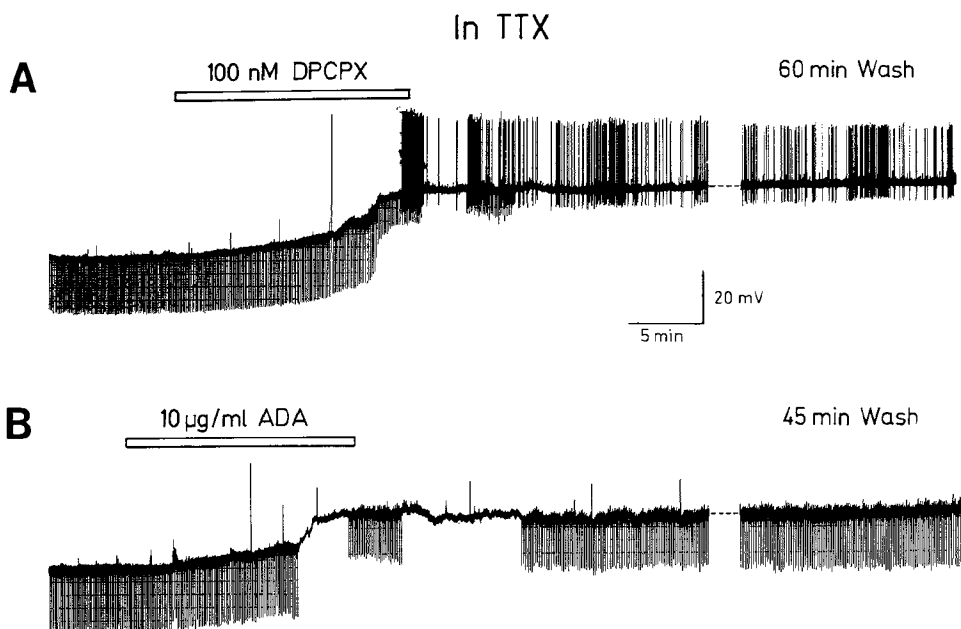


Fig. 4. Postsynaptic actions of DPCPX and ADA on TTX-treated CA3 pyramidal cells. Chart records of the membrane potential from two different CA3 neurons (A: resting membrane potential  $-66\text{ mV}$ ; input resistance  $100\text{ M}\Omega$ ; B: resting membrane potential  $-62\text{ mV}$ ; input resistance  $105\text{ M}\Omega$ ). The input resistances were monitored by repetitive injection of hyperpolarizing current pulses (0.2 nA, 500 ms). The addition of DPCPX (A) or ADA (B) to a TTX-containing bathing solution led to a gradual depolarization which eventually reached the threshold for TTX-insensitive spikes (presumably calcium spikes, A). The drug-induced depolarizations were irreversible.

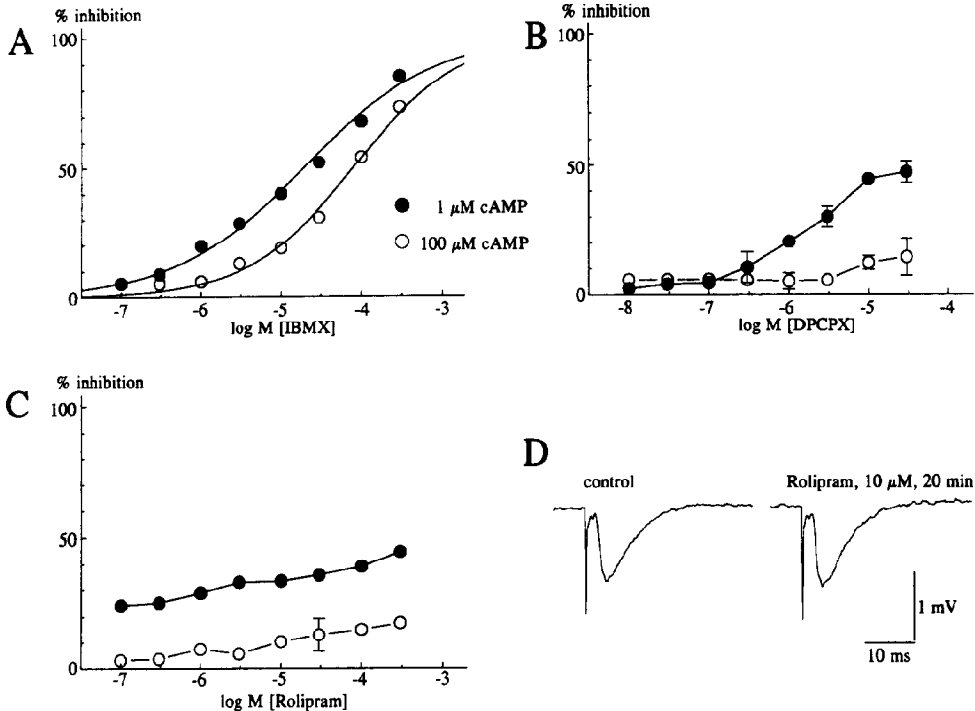


Fig. 5. Action of DPCPX on total cAMP phosphodiesterase activity and electrophysiological effects of rolipram. A–C show the percentage inhibition of total cAMP phosphodiesterase activity as a function of the concentration of IBMX (A), DPCPX (B) and rolipram (C). The enzyme activity was measured in a homogenate from rat cerebral cortex using a radiochemical assay. The tests were performed either in the presence of 1  $\mu$ M cAMP (filled circles) or 100  $\mu$ M cAMP (open circles). The data in A were fitted by a sigmoid function. Each point represents the mean value of nine measurements. When error bars are lacking, the standard deviation was smaller than the size of the symbol. In D, the effects of rolipram (10  $\mu$ M, 20 min) on stimulus-evoked fEPSPs recorded extracellularly in stratum radiatum of area CA3 are shown. The field potentials were elicited by stimulation of the mossy fiber pathway using half-maximal stimulus intensity. The time point of stimulation is indicated by the stimulus artifact. Each trace represents the average of five consecutive recordings.

when DPCPX concentrations greater than 10  $\mu$ M were added to the assay, the effect saturated at a 45–50% inhibition of total phosphodiesterase activity (Fig. 5B). In the presence of 100  $\mu$ M cAMP, the inhibitory potency of DPCPX was found to be significantly smaller. In this case, 30  $\mu$ M DPCPX blocked only about 15% of total phosphodiesterase activity. At the concentrations used in electrophysiological experiments (up to 100 nM), DPCPX suppressed total phosphodiesterase activity by about 5%, independent of substrate concentration (Fig. 5B). The actions of DPCPX on total phosphodiesterase activity resembled those of the selective phosphodiesterase inhibitor rolipram.<sup>10</sup> This pyrrolidone derivative, which displays no affinity to adenosine receptors, selectively inactivates the cAMP-specific isozyme (PDE IV<sup>11</sup>), which is characterized by a high affinity to cAMP ( $K_m$ : 2  $\mu$ M<sup>10</sup>). Similar to DPCPX, the inhibitory potency of rolipram was higher at a substrate concentration of 1  $\mu$ M than at 100  $\mu$ M cAMP, and the effect seemed to saturate at about 40–50% inhibition of total phosphodiesterase activity (Fig. 5C). However, in

contrast to DPCPX, even at low concentrations (0.1–1  $\mu$ M), rolipram blocked 25–30% of the enzyme activity (Fig. 5C).

Since higher concentrations of DPCPX suppressed phosphodiesterase activity, we investigated whether the phosphodiesterase inhibitor rolipram would mimic the effects of DPCPX on fEPSPs recorded extracellularly in the stratum radiatum of area CA3.<sup>4</sup> Rolipram was applied at a concentration (10  $\mu$ M) that inhibited about 30% of the total phosphodiesterase activity (Fig. 5C). In all slices tested ( $n = 4$ ), rolipram did not significantly change the amplitude and the rise time of the evoked field potentials (Fig. 5D). In no case were spontaneous epileptiform discharges observed.

#### DISCUSSION

The physiological role of ambient adenosine concentrations in the hippocampal CA3 network was investigated using the selective adenosine A<sub>1</sub> receptor antagonist DPCPX. Brief application of the drug induced a prolonged disinhibition of CA3 neurons

associated with epileptiform burst discharges in more than 90% of all neurons tested. Since other, more trivial explanations of this phenomenon could be excluded—i.e. irreversible A<sub>1</sub> receptor blockade or persistence of the drug in the slice (see Fig. 3 in Ref. 4), or side effects of DPCPX on phosphodiesterase activities (Fig. 5)—it appears legitimate to assume that the sustained up-regulation of excitability is causally linked to the transient suppression of endogenous adenosinergic inhibition. This notion was further supported by the observation that the phosphodiesterase inhibitor rolipram, which lacks adenosine receptor affinity, failed to mimic the actions of DPCPX (Fig. 5), while the adenosine-degrading enzyme ADA produced very similar effects (Ref. 7 and Fig. 4).

#### *Suppression of adenosine tonus leads to synaptically mediated burst discharges*

With respect to the origin of the DPCPX-induced burst discharges, several observations indicate that they were synaptic in nature: while both the shape and the frequency of intrinsically generated discharges of CA3 pyramidal neurons are strictly voltage-dependent,<sup>27,28</sup> DPCPX-induced bursts occurred independently of the pre-burst membrane potential. Furthermore, the amplitude of the slow depolarization underlying the PDS increased with hyperpolarization, indicating that the burst discharges represented synaptically mediated events resembling so-called giant EPSPs.<sup>30</sup> Consequently, DPCPX failed to induce burst discharges when glutamatergic excitatory synaptic transmission was blocked by adding the amino-3-hydroxy-5-methyl-4-isoxazolpropionic acid receptor antagonist CNQX to the bathing solution. Several recent findings from other laboratories lend support to the hypothesis that the DPCPX-induced epileptiform activity is mainly due to a disinhibition of excitatory transmitter release: (1) In a recent quantal analysis of glutamatergic transmission in the hippocampal slice, Prince and Stevens have demonstrated that adenosine reduces excitatory neurotransmitter release, while relatively low concentrations of the non-specific adenosine antagonist IBMX alone significantly increase the amount of excitatory transmitter released.<sup>41</sup> Therefore, Prince and Stevens have suggested that the resting level of endogenous adenosine is sufficient to tonically reduce synaptic strength. (2) In organotypic slice cultures of the hippocampus, adenosine antagonists increased both the frequency and size of spontaneous glutamatergic EPSPs recorded in the presence of the GABA<sub>A</sub> receptor antagonist bicuculline.<sup>40</sup> (3) In the presence of adenosine antagonists, an increase in basal and stimulus-evoked release of glutamate from cultured cerebellar granule cells has been demonstrated.<sup>40</sup>

Interestingly, the induction by DPCPX of sustained bursting activity seems to require intact glutamatergic transmission, since no epileptiform activity

was observed after application of DPCPX in the presence of CNQX and subsequent washout of both drugs (Fig. 2). This suggests that the mechanism initiating the long-term up-regulation of excitability is in some way activity-dependent. It is, for example, conceivable that the large calcium influxes that accompany PDS-like discharges might be needed as a co-factor in the process that eventually leads to self-sustained hyperexcitability.<sup>38</sup> This hypothesis is also supported by earlier field potential recordings in CA1 "minislices", in which area CA3 was surgically removed. In this preparation, 100 nM DPCPX consistently failed to induce stimulus-evoked or spontaneous epileptiform activity, but was capable of enhancing the amplitude of the population spike by 40%.<sup>4</sup> When using a paired-pulse paradigm, DPCPX was found to reduce the facilitation ratio (amplitude of test pulse divided by amplitude of conditioning pulse) by about 25% in this preparation, suggesting enhanced transmitter release (unpublished observation). However, in contrast to its long-lasting effect on synaptic transmission in the CA3 network, this action of DPCPX in CA1 "minislices" was slowly reversible within 60–90 min of drug washout. Again, this discrepancy might be ascribed to the fact that the initiation of a self-sustained process requires very strong excitatory activity as provided by the PDSs seen in area CA3, but absent in CA1 "minislices". In addition, regional differences in receptor density or location, or in receptor coupling to effector systems, could also contribute to the differential action of DPCPX in isolated area CA1 vs CA3 of hippocampus.

#### *Postsynaptic component of adenosine tonus*

Experiments performed in the presence of TTX or the non-NMDA glutamate antagonist, CNQX, revealed an additional strong postsynaptic component of endogenous adenosinergic inhibition. On the postsynaptic site, removal of the adenosine tonus produced a prolonged depolarization of the membrane potential accompanied by an increase in spontaneous firing (in CNQX) or by an appearance of TTX-insensitive, presumed calcium spikes (in TTX). The occurrence of TTX-insensitive action potentials at depolarized membrane potentials is quite a common phenomenon in CNS neurons and can be observed in the presence of TTX-containing bath solutions without any convulsive drug.<sup>33</sup> Therefore, it is legitimate to conclude that postsynaptic calcium currents underlying these calcium spikes probably contribute to the shape and the magnitude of a DPCPX-induced PDS, but a facilitation of these currents is unlikely to cause the DPCPX-evoked epileptiform activity.

#### *Possible mechanisms of sustained hyperexcitability after transient suppression of adenosine tonus*

As a xanthine derivative, DPCPX might also act on brain phosphodiesterases. In fact, measurements of the effects of DPCPX on total cAMP



phosphodiesterase activity in rat cerebral cortex revealed such an action. However, the concentrations of DPCPX necessary to produce a significant block of phosphodiesterase activity ( $>300$  nM) are two to three orders of magnitude higher than those which are sufficient to half-maximally inhibit adenosine  $A_1$  receptors. In binding studies, the  $K_i$  of DPCPX for displacing [ $^3$ H]cyclohexyladenosine from  $A_1$  receptors was found to be 0.46 nM<sup>13</sup> and electrophysiological measurements applying Schild plot analysis revealed a  $K_D$  value of 3.3 nM.<sup>2</sup> In the electrophysiological experiments described in this study, the concentrations of DPCPX never exceeded 100 nM. Therefore, we conclude that under the given experimental conditions, the changes in neuronal properties observed following application of DPCPX are solely due to a selective blockade of adenosine  $A_1$  receptors.

Can the functionally significant adenosine tonus found in the hippocampal slice preparation represent an artifact due to the non-specific accumulation of extracellular adenosine resulting from cell damage during the dissection and slicing procedure, and the slow exchange rate between the core of the slice and the surrounding bathing solution? This hypothesis has recently been proposed by Thompson *et al.*, who failed to observe intrinsic excitatory actions of DPCPX in CA3 neurons of an organotypic slice culture.<sup>52</sup> Although the preparation of slices will inevitably be accompanied by an increase in extracellular adenosine, the following observations indicate that this increase should remain transient and hence not introduce a substantial bias to our study.

(1) Measurements of ambient adenosine levels in slice preparations and in the CNS of intact animals have yielded very similar values.<sup>22,56</sup> (2) Methylxanthines produce a variety of excitatory actions in intact animals which are generally believed to be due to their antagonism of endogenous adenosine.<sup>49</sup> In addition, the organotypic slice might not be the most suitable preparation to study endogenous adenosinergic inhibition. During weeks of culturing, slices of CNS tissue are reduced to a monolayer of cells. It is thus conceivable that the perfusion system required to change bathing solution and apply drugs might wash away any endogenously released adenosine. Furthermore, the strong recurrent glutamatergic network of area CA3, which mediated the DPCPX-triggered, sustained PDS-like discharges in our preparation, might be less developed and elaborate in a monolayer of organotypically cultured cells.

In view of the strong excitatory coupling of CA3 neurons,<sup>37</sup> it can be argued that the DPCPX-induced burst discharges, once initiated, might be sustained by reverberating and oscillating excitations so that the persistent epileptiform activity would represent a network phenomenon rather than a drug-induced alteration in the level of excitability. If so, other manipulations leading to seizure activity in area CA3 should also be followed by self-sustained

epileptiform discharges. However, spontaneous epileptiform activity induced either by changes in the ionic composition of the bathing solution (increased potassium concentration, removal of magnesium ions) or by kainate completely ceased after switching back to control solution.<sup>12,15</sup> In contrast to their lacking effect on post-drug spontaneous activity, both high extracellular potassium concentration and kainate produce a long-lasting disinhibition of the mossy fiber-CA3 cell synapse, the initiation of which can be prevented by the NMDA receptor antagonist D-APV.<sup>12</sup> This antagonist, however, did not block the induction of spontaneous and stimulus-evoked burst discharges by DPCPX, indicating that activation of NMDA receptors is not required for the long-term effects of DPCPX to develop.

How can the transient suppression of an adenosine tonus lead to a sustained increase in neuronal activity? In central neurons, three different  $A_1$  receptor-mediated actions of adenosine have been identified so far, namely activation of a potassium conductance,<sup>1,23</sup> decrease of a calcium conductance,<sup>42</sup> and inhibition of adenylate cyclase.<sup>54</sup> The closure of potassium channels which are tonically activated by endogenous adenosine might account for the early depolarization following application of DPCPX. With respect to the long-lasting effects of the  $A_1$  antagonist, facilitation of calcium influx and/or disinhibition of adenylate cyclase could serve as possible mediators that initiate a self-sustained process. Rises in cytosolic long-term or cAMP have indeed been implicated in long-term increases in hippocampal excitability.<sup>21,35,39a,50</sup>

With respect to a putative increase in cAMP, one might expect that the drug would simultaneously decrease the amplitude of the afterhyperpolarization, which has previously been demonstrated to accompany an increase in intracellular cAMP.<sup>34a</sup> This effect, however, was not observed following DPCPX application. Instead, the drug (100 nM) was found to slightly enhance the amplitudes of both the fast and slow afterhyperpolarization components (unpublished observation). This effect might be due to the DPCPX-induced increase in input resistance. It should be noted, however, that the result of this experiment does not rule out an increase in cytosolic cAMP, because other substances known to affect cAMP levels also failed to decrease afterhyperpolarization amplitudes in hippocampus.<sup>50</sup> What mechanism can account for the apparently variable action of intracellular cAMP rises on afterhyperpolarization amplitudes? A possible explanation would be that DPCPX-induced increases in cAMP occur locally in restricted compartments of the neuron and thus do not affect the conductance mediating the afterhyperpolarization, while transmitters that alter afterhyperpolarization amplitude via cAMP elevation produce rises of this second messenger in close proximity to the corresponding ion channels.

## CONCLUSIONS

Our results indicate that endogenous adenosine acting on A<sub>1</sub> receptors exerts a strong control of both the pre- and postsynaptic excitability in the hippocampal CA3 region. A transient suppression of the inhibitory tonus causes sustained epileptiform burst discharges that are synaptic in nature, but do not require initial NMDA receptor activation to persist. This long-lasting disinhibition most likely involves one or both of the signaling pathways modulated by endogenous adenosine, namely calcium influx and/or cytosolic cAMP formation. For the moment, our data do not allow us to

determine how and to what extent the different A<sub>1</sub> receptor-mediated pathways contribute to the long-term effects observed after a transient removal of endogenous adenosinergic inhibition. Further research will be required to explain the delicate relationship between adenosine tonus and neuronal excitability in terms of its molecular machinery.

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