New Type of Synaptically Mediated Epileptiform Activity Independent of Known Glutamate and GABA Receptors

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Skov, Jane, Steen Nedergaard, and Mogens Andreasen. New type of synaptically mediated epileptiform activity independent of known glutamate and GABA receptors. J Neurophysiol 93: 1845–1856, 2005. First published November 10, 2004; doi:10.1152/jn.00656.2004. It is well known that excitatory synaptic transmission at the hippocampal CA3–CA1 synapse depends on the binding of released glutamate to ionotropic receptors. Here we report that during long-term application of Cs⁺ (5 mM), stimulation of the Schaffer collateral-commissural pathway evokes an epileptic field potential (Cs-FP) in area CA1 of the rat hippocampal slice, which is resistant to antagonists of ionotropic glutamate and GABAergic receptors. The Cs-FP was blocked by N-type but not L-type Ca²⁺ channel antagonists and was attenuated by adenosine (0.5 mM), as expected for a synaptically mediated response. These properties make the Cs-FP fundamentally different from other types of Cs⁺-induced epileptiform activity. Replacement of Cs⁺ with antagonists of the hyperpolarization-activated nonselective cation current Iₖ, and inwardly rectifying potassium channels (KIR) or partial inhibition of the Na⁺/K⁺ pump did not cause Cs-FP-like potentials, which indicates that such actions of Cs⁺ were not responsible for the Cs-FP. The effect of Cs⁺ was partly mimicked by 4-aminopyridine (4-AP; 2 mM), suggesting that an increase in transmitter release is involved. The group I metabotropic glutamate receptor (mGluR) agonist (RS)-3,5-dihydroxyphenylglycine (DHPG) attenuated the Cs-FP. This effect was not, however, antagonized by group I mGluR antagonists. Selective and nonselective mGluR antagonists did not attenuate the Cs-FP. We conclude that long-term exposure to Cs⁺ induces a state where excitatory synaptic transmission can exist between area CA3 and CA1 in the hippocampus, independent of ionotropic and metabotropic glutamate receptors and GABAₐ receptors.

INTRODUCTION

Epilepsy covers a diverse group of disorders of brain function, where the characteristic feature is the occurrence of unprovoked epileptic seizures resulting from excessive discharge in a population of hyperexcitable neurons. Most epileptic seizures are due to discharges generated in cortical and hippocampal structures (Avanzini and Franceschetti 2003) and is believed to derive from an imbalance between hyperpolarizing and depolarizing influences in an interconnected network of neurons (McCormick and Contreras 2001). There seems to be no single cause for this imbalance, which can arise as a result of changes in the properties and/or density of ion channels (Na⁺, Ca²⁺, or K⁺ channels) or through altered glutamatergic, GABAergic, or nicotinergic neurotransmission (for review, see Avanzini and Franceschetti 2003). However, the changes that account for the most common forms of epilepsies have so far not been clarified. Experimentally, imbalance in cortical circuits can be induced by a variety of methods that has resulted in the development of several types of in vivo and in vitro models of epileptogenesis. In nearly all of these models, the generation of epileptic activity depends on neurotransmission (McCormick and Contreras 2001). In in vivo models, excitatory synaptic transmission is strengthened either through electrical stimulation (kindling) or pharmacologically through injection of e.g., kainic acid or pilocarpine. In most in vitro models, excitatory synaptic transmission is strengthened either directly by enhancing N-methyl-d-aspartate (NMDA) receptor–mediated synaptic transmission (Mg²⁺-free model) or increasing transmitter release (4-AP model), or indirectly, by reducing GABAergic inhibition or the efficacy of hyperpolarizing K⁺ currents (high K⁺ model) (for review, see Avanzini and Franceschetti 2003; Löscher 2002; McCormick and Contreras 2001). Spontaneous epileptic activity can, however, also be seen as a result of enhanced GABAergic activity (Uusisaari et al. 2002), and even in low extracellular Ca²⁺ conditions, where synaptic transmission altogether is greatly reduced or even blocked (Jefferys and Hass 1982).

In some forms of epilepsy, interictal spikes, which are brief, large, sharp spikes in the EEG, are observed between seizures. These interictal spikes are the result of synchronized activation of a large population of neurons in local cortical circuits and is commonly observed in both acute and chronic models of epileptogenesis (de Curtis and Avanzini 2001). Studies on in particular in vitro models have clearly indicated that the synchronization of neurons during interictal activity is dependent on activation of glutamatergic AMPA and NMDA receptors (for review, see de Curtis and Avanzini 2001; McCormick and Contreras 2001). However, in this paper, we present a novel type of interictal-like activity, induced in the hippocampus by extracellular application of low concentration of cesium (Cs⁺), which does not involve AMPA or NMDA receptors.

It is well established that Cs⁺ can induce spontaneous epileptiform activity in hippocampal and neocortical brain slices (D’Ambrosio et al. 1998; Hwa and Avoli 1991; Janigro et al. 1997; Xiong and Stringer 1999, 2001). However, no conclusive evidence has been put forward regarding the specific mechanism(s) behind the epileptogenic effect of Cs⁺. Cs⁺ has a number of different actions on neurons and astrocytes, including blockade of the inwardly rectifying potassium channel, KᵢR (Ransom and Sontheimer 1995), blockade of the hyperpolarization-activated h channel (Maccabarri et al. 1993), interference with the Na⁺/K⁺ pump (Aker et al. 1979; Sachs 1977), and increasing quantal transmitter release (Kuma-
moto and Kuba 1985). In some studies, Cs⁺-induced epileptic activity has been reported to depend on an increased activation of ionotropic glutamate receptors (Hwa and Avoli 1991; Xiong and Stringer 1999), which could indicate that an altered synaptic transmission is of importance in Cs⁺-induced epileptic activity. On the other hand, Cs⁺ has also been shown to induce epileptic activity under conditions where synaptic transmission is blocked (Xiong and Stringer 2001), which points to the possible involvement of nonsynaptic mechanisms. The Cs⁺-induced epileptiform activity presented here is not dependent on increased activation of ionotropic glutamate or GABA_A receptors, even though it has all the characteristics of synaptically mediated activity. The mechanism behind this Cs⁺-induced response therefore seems to differ fundamentally from what has so far been suggested as plausible causes not only for Cs⁺-induced epileptic activity but for epileptiform interictal activity in general. The aims of this study were, therefore, to characterize this novel type of in vitro interictal-like activity, to determine the relative contribution from synaptic and nonsynaptic elements, and to gain insight into the specific mechanism through which Cs⁺ generates epileptiform activity.

METHO DS

All experimental protocols were in accordance with university guidelines for animal research and complied with Danish and European law on the care and use of laboratory animals. Experiments were performed on hippocampal slices prepared from 221 male Wistar rats (250–300 g). The rats were anesthetized with either chloroform or isoflurane and decapitated. The brain was removed and quickly placed in a dissection medium (see Drugs and solutions) at 4°C. The hippocampus was dissected free, and 400-μm slices were cut on a McIlwain tissue chopper. One slice was immediately transferred to the recording chamber, where it was placed on a nylon-mesh grid at the interface between warm (31–33°C) standard Ringer solution (see recording chamber, where it was placed on a nylon-mesh grid at the border between area CA3 and area CA1 was used to stimulate orthodromically the Schaffer collateral-commissural fibers with constant current pulses (50 μA, 0.15–0.4 mA) at a frequency of 0.05 Hz.

Conventional recording techniques were employed using a high-input impedance amplifier (Axoclamp 2A, Axon Instruments) with bridge balance and current injection facilities. Signals were digitized on-line via a Labmaster AD/CD converter and pCLAMP acquisition software (Axon Instruments) on a 486 PC and recorded on videotape using a modified digital audio processor (Sony PCM-701es) for off-line analysis. Signal analyses were performed using pCLAMP analysis software.

Slices were accepted if, during control conditions, they displayed a normal orthodromic field potential [defined as a field excitatory postsynaptic potential (fEPSP) with a single population spike of a maximal amplitude between 5 and 10 mV] and showed no additional spikes with supramaximal stimulation. As has been reported by others (Xiong and Stringer 1999), spreading depressions occurred often after long-term exposure to Cs⁺. Following a spreading depression, the stimulus-evoked response was monitored closely and recordings were stopped if the baseline potential and field potential did not fully recover or if spreading depressions occurred three times. With those criteria, we found no evidence that spreading depression influenced the outcome of pharmacological treatments. Specifically, we compared the effects of (RS)-3,5-dihydroxyphenylglycine (DHPG) and adenosine (see RESULTS) in slices with and without spreading depression. For both compounds, every value obtained with spreading depression was within the 95% limits of the mean value obtained without spreading depression (other drug responses were not tested, either because spreading depression did not occur or the drug had no effect). Although the sensitivity of the above test is limited, it shows that spreading depression is unlikely to affect the results to any large degree (if at all), and the data with one or two spreading depression episodes were therefore included in the analyses. Inclusion of such data seems further justified when considering that spreading depression, occurring in slices under normal metabolic conditions, has been shown to be fully reversible with respect to a number of key parameters in the slice, i.e., the volume of extracellular space, the DC voltage, the extracellular concentrations of K⁺ and Ca²⁺, and pH (Herreras and Somjen 1993; Jing et al. 1994; Obediat and Andrew 1998; Tong and Chesler 2000).

An estimate of the size of the fEPSP and Cs-FP was obtained as the difference between the peak of the field potential and the prestimulation baseline potential. The occurrence of population firing during the rising phase of the field potential precluded measurements of the rate-of-rise. The duration of the fEPSP and the Cs-FP was measured from the onset of the positive component to the time where the potential reached or crossed the prestimulation potential.

For statistical analysis of pharmacological effects, the paired t-test was used. For each group of experiments, the values obtained in the presence of an applied compound were compared with the control values before the application. This testing method was also applied in the cases where multiple treatments were presented in the same graph as in Fig. 9. Here it should be noted that the statistical evaluation did not involve a comparison between different treatments: each group of measurement was considered a separate experiment, and the values obtained during individual treatments were presented and tested only with respect to its own set of control values. In every case, the two-tail test was applied, and the level of significance was set at 5% (degrees of freedom equals n – 1). n denotes the number of slices used and for each experimental approach slices from more than one animal were included. To test if the use of multiple slices from the same animal rather than from different animals could bias the results, we compared the control amplitude of the Cs-FP in 20 slices from the five rats where 4 slices were used from each. ANOVA test on this material showed that the estimated variance (mean square) among rats was only slightly higher than that of individual determinations. This difference was not statistically significant (F = 1.13; df = 4 and 15, respectively). A similar result was obtained for the Cs-FP duration in the same 20 slices. From these findings we concluded that the use of more slices from the same rat will not introduce a bias on the basic parameters of the Cs-FP. Therefore for each treatment, data from individual slices were pooled irrespective of the number of rats used. Values are given as means ± SE unless otherwise indicated.

Drugs and solutions

The composition of the dissection medium was (in mM) 120 NaCl, 2 KCl, 1.25 NaH₂PO₄, 6.6 HEPES acid, 2.6 NaHEPES, 2.6 NaHCO₃, 10 d-glucose, 2 CaCl₂, and 2 MgSO₄, bubbled with carbogen. The composition of the standard Ringer solution was (in mM) 124 NaCl, 3.25 KCl, 1.25 NaH₂PO₄, 20 NaHCO₃, 2 CaCl₂, 2 MgSO₄, and 10 d-glucose, bubbled with carbogen (pH 7.3). In experiments where Cd²⁺ or Ba²⁺ was applied, a standard Ringer solution was used in which phosphate and sulfate had been omitted to prevent precipitation.

Unless otherwise noted, the experiments were all performed in the presence of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 μM),...
dl-2-amino-5-phosphonopentanoic acid (APV, 50 µM), and bicuculline methoh bromide (10 µM). In most experiments, (+)-baclofen (20 µM) or the racemic mixture (40 µM) was also included to dampen background oscillatory activity.

Most pharmacological compounds were made up in aqueous stock solutions of 100–1,000 times the required final concentration and diluted in the Ringer solution as appropriate. (2S)-2-amino-2-[(1S,2S)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl)propanoic acid (LY341495), 2-methyl-6-(phenylethyl)pyridine hydrochloride (MPEP), and nifedipine were dissolved in DMSO. The final concentration of DMSO was maximally 0.5%, which was found to have no effect when applied alone. Experiments with light-sensitive compounds [nifedipine and 4-ethylphenylamino-1,2-dimethyl-6-methylaminopyrimidinium chloride (ZD7288)] were performed in a dark environment.

For application of ω-conotoxin GVIA, a glass micropipette (tip diameter, 10 µm) was filled with 40 µM ω-conotoxin GVIA in isotonic NaCl solution. The tip of the pipette was placed above the surface of the slice close to and upstream to the recording electrode, and a drop was applied to the surface of the slice. After application, the pipette was immediately withdrawn.

RESULTS

Characterization of a Cs⁺-induced field potential

In slices perfused with standard Ringer solution, stimulation of the Schaffer collateral-commisural fibers gave a normal orthodromic fEPSP with one population spike (Fig. 1A). The average amplitude of the fEPSP was 2.7 ± 0.08 mV, and the duration was 24.8 ± 0.6 ms (n = 230). As expected, co-application of CNQX (10 µM), APV (50 µM), and bicuculline (10 µM) to block glutamatergic non-NMDA and NMDA receptors and GABA_A receptors, respectively, completely abolished this field potential (Andreasen et al. 1989). However, in the presence of these antagonists, application of Cs⁺ (5 mM) consistently led to the development of a new field potential in response to stimulation of the Schaffer collateral-commisural fibers (Fig. 1B). This potential was characterized by a remarkably slow induction phase. Thus it started to appear after a wash-in period of 30–60 min and subsequently increased gradually until it reached a maximal size, typically after 40–70 min of perfusion with Cs⁺ (Fig. 1B).

When fully developed, the Cs-FP was easily distinguished from a normal orthodromic field potential on the basis of its shape and duration. It consisted of two phases: an initial positive component with a peak amplitude of 5.7 ± 0.2 mV and a duration of 145.1 ± 5.5 ms (n = 228) followed by a longer-lasting (up to several seconds) negative component (Fig. 1B). A short burst of population spikes (mean, 3.3 ± 0.08 spikes) was always observed at the onset of the potential (Fig. 1B). In several experiments, synchronized firing activity was also seen during the later parts of the potential (Fig. 5D). The transition between the positive and negative component was usually smooth, except in 37% of the recordings, where the rate of decay at this point showed a transient decrease, giving the appearance of a “shoulder” (Fig. 1B). In experiments where the glutamate receptor antagonists had been omitted, a Cs-FP still developed, indicating that glutamate receptor blockade is not necessary for its induction.

Recurrences excitatory synapses have been shown between CA3 pyramidal neurons (Miles and Wong 1986). To test whether recruitment of CA3 networks was necessary for the induction of Cs-FP, the CA3 area was removed from five slices before wash-in of Cs⁺. In all slices, Cs-FPs developed, which were qualitatively similar to those evoked in intact slices. Because Cs⁺ has been shown to increase quantal transmitter release (Kumamoto and Kuba 1985), and since CNQX and APV are both competitive antagonists, it is conceivable that the Cs-FP could be caused by a displacement of CNQX and/or APV from their receptors. To examine whether the effect of Cs⁺ was due to insufficient block of the ionotropic glutamate receptors, the concentration of APV was increased to 200 µM either alone (n = 3) or in combination with an increased concentration of CNQX to 20–25 µM (n = 6). Neither of these treatments had any appreciable effect on the Cs-FP. In a further 15 experiments, these high concentrations of CNQX and APV were present from the onset of the experiments. The Cs-FPs observed under these conditions were indistinguishable from those seen in lower concentrations of the antagonists. The noncompetitive NMDA receptor antagonist MK-801 (10 µM)
also had no effect on the Cs-FP \( (n = 8) \). These results suggested that the Cs-FP is unlikely to be due to glutamate release onto CNQX- or APV-sensitive glutamate receptors.

Previous reports have shown that Cs\(^+\) induces spontaneous epileptic activity in slices treated with bicuculline alone (Hwa and Avoli 1991). To investigate whether blockade of synaptic inhibition was necessary for the development of the Cs-FP, we omitted bicuculline in some of the experiments \( (n = 11) \). This treatment did not prevent the Cs-FP. However, the average amplitude and duration of the positive component was somewhat reduced in the absence of bicuculline. Subsequent addition of bicuculline \( (10\mu M) \) increased the response in three of three experiments. An increase in the concentration of bicuculline from 10 to 60 \( \mu M \) had no further effect \( (n = 5) \). These experiments therefore indicated that blockade of GABAergic inhibition is not a prerequisite for the development of the Cs-FP.

Since the Cs-FP depended on stimulation, we investigated the relationship between stimulation intensity and the amplitude of the Cs-FP. The threshold, defined as the lowest stimulation intensity that evoked a detectable field potential (average peak amplitude: 0.35 mV; range, 0.1–0.8 mV), was found to vary between 30 and 100 \( \mu A \) \( (n = 5) \). For the construction of input-output curves, the field potentials evoked at each stimulation intensity were normalized with respect to the peak amplitude at \( 2 \times \) Thr.

FIG. 2. Cs-FP is activated in an all-or-nothing fashion. A: Cs-FP evoked with stimulation intensities of 30, 40, and 60 \( \mu A \). In this experiment, 30 \( \mu A \) was identified as the threshold (Thr.) value. B: histogram of the normalized peak amplitude \( (n = 5) \) of the initial positive component in relation to stimulation intensity. Measurements were normalized with respect to the peak amplitude at \( 2 \times \) Thr.

At high stimulation frequencies, both the initial and the late component declined in amplitude, and the duration of the late component was reduced \( (Fig. 3A) \). In regard to the initial component, no changes were seen in the range of frequencies between 0.05 and 0.2 Hz \( (Fig. 3B) \). At frequencies \( \geq 0.2 \) Hz, there was a progressive decline in the peak amplitude of both the positive and the negative component, suggesting that the Cs-FP has a relative refractory period of about 5 s.

A spatial profile of the Cs-FP along the somatodendritic axis of the CA1 pyramidal neurons was obtained from recordings taken at 100-\( \mu m \) intervals along a line perpendicular to s. pyramidalis from the alveus to the hippocampal fissure as outlined in Fig. 4A. Measurements of the amplitudes of the two components were made as exemplified in Fig. 4B. The summary plot from five experiments shows that the initial component had maximal positivity in s. pyramidalis and maximal negativity in s. radiatum \( (Fig. 4C) \). A similar distribution of positivity and negativity was found for the control fEPSP \( (n = \)...
1, data not shown) and is in agreement with that expected for orthodromic stimulation of the Schaffer collateral-commisural fibers in s. radiatum (Andersen et al. 1966). The observed spatial profile of the initial component is therefore compatible with an origin of the Cs-FP from synapses located in s. radiatum. The late component measured 1,000 ms after the stimulation was negative at all recording sites with maximal amplitude in s. pyramidalis and proximal s. radiatum (Fig. 4 C).

Because of the clear relationship between the size of the positive and the negative component and the fact that the negative component never occurred in isolation, we focused our attention on the positive component.

**Possible synaptic origin of the Cs-FP**

We extended the investigation of the possible synaptic nature of the Cs-FP by testing its sensitivity to manipulations of synaptic transmission. In the six slices examined, the Cs-FP was abolished by perfusion with nominally Ca\(^{2+}\)-free medium containing 6 mM Mg\(^{2+}\) (Fig. 5A), and full recovery was obtained following reintroduction of 2 mM Ca\(^{2+}\) (data not shown). Perfusion with the nonspecific Ca\(^{2+}\) channel antagonist Cd\(^{2+}\) (0.2–0.4 mM, n = 7) also abolished the Cs-FP (Fig. 5B). Drop application of the N-type Ca\(^{2+}\) channel antagonist \(\omega\)-conotoxin GVIA (40 \mu M) significantly reduced the average amplitude of the response by 82.4 ± 13.4% (P = 0.02, n = 5, Fig. 5C). As a control for this effect, we observed that in normal medium, drop application of \(\omega\)-conotoxin GVIA reduced the amplitude of intracellularly recorded EPSPs by on average 75.2 ± 13.6% (results not shown), which is similar to the efficiency of bath application of 1 \mu M \(\omega\)-conotoxin GVIA (Luebke et al. 1993; Wu and Saggau 1994). The L-type Ca\(^{2+}\) channel antagonist nifedipine (10 \mu M) was also tested but found to have no effect in any of four slices tested. Together, these data support a synaptic origin of the Cs-FP. To further test this idea, we examined the effect of adenosine that has been shown to inhibit presynaptic glutamate release in area CA1 (Yoon and Rothman 1991). Adenosine (0.5 mM) gave a marked reduction of the Cs-FP, on average by 77.7 ± 14.1% (P < 0.01, n = 7, Fig. 5D). The effect of adenosine was reversible.

As mentioned in METHODS, baclofen (20 \mu M) was included in the perfusion medium in the majority of experiments (n = 169) to dampen spontaneous oscillatory activity. Baclofen, like adenosine, is known to reduce presynaptic glutamate release (Dumas and Foster 1998; Wu and Saggau 1997; Yoon and Rothman 1991) and would therefore be expected to reduce the...
Cs-FP, as seen with adenosine. However, the Cs-FP was unaffected by application of baclofen even at concentrations of \( \pm 100 \) \( \mu \text{M} \) (\( n = 3 \)). Control experiments in normal medium showed that baclofen (20 \( \mu \text{M} \)) reduced the normal fEPSP by 62.9 \( \pm \) 13.6\% (\( P < 0.01, n = 6, \text{Fig. 6} \)), indicating that the concentration used was effective. Interestingly, however, we also observed that short-term perfusion with Cs\(^+\) or Ba\(^{2+}\) (10–15 min) reversed this latter effect of baclofen on the normal fEPSP (Fig. 6, \( n = 6 \)). It therefore seems possible that such antagonistic effect of Cs\(^+\) could explain why baclofen had no effect on the Cs-FP.

We also considered whether ephaptic interactions or field effects could be involved in the Cs-FP. Since such nonsynaptic interactions between neurons are known to be very sensitive to changes in extracellular osmolarity (Schwartzkroin et al. 1998), we tested the effect of adding 30 mM sucrose to the perfusion medium. This treatment reduced the amplitude of the positive component of the Cs-FP by 15.6 \( \pm \) 7\% (\( n = 5, P = 0.06 \)) of the original value (data not shown). In a control experiment, 30 mM sucrose gave a reduction in the amplitude of a normal orthodromic fEPSP by 17.5\% of its original value. In comparison, the observations suggested that the Cs-FP is no more affected by increased extracellular osmolarity than what would be expected for a normal synaptically mediated field potential.

Mechanisms of action of Cs\(^+\)

To examine more closely which of the numerous effects of Cs\(^+\) are responsible for the development of the Cs-FP, we sought to mimic these effects either individually or in combination. External application of 2 mM Cs\(^+\) has been shown to block the hyperpolarization-activated current, \( I_{\text{h}} \), in CA1 hippocampal neurons (Maccarelli et al. 1993), an effect that could theoretically explain our observations. We therefore tested if the replacement of Cs\(^+\) with ZD7288 (20 \( \mu \text{M} \)), a specific antagonist of \( I_{\text{h}} \) (Gasparini and DiFrancesco 1997), would have a similar effect. However, this treatment did not result in the induction of any Cs-FP–like responses within a 40- to 75-min observation period (\( n = 3, \text{Fig. 7A} \)).

The inwardly rectifying K\(^+\) channel, \( K_{\text{IR}} \), which shows high expression in astrocytes, is also sensitive to Cs\(^+\) (Ransom and Sontheimer 1995; Sontheimer and Waxman 1993). A plausible mechanism for the induction of the Cs-FP could be accumulation of extracellular K\(^+\) secondary to blockade of this channel. To test this hypothesis, we applied Ba\(^{2+}\) (0.2 mM), which at this low concentration primarily antagonizes the \( K_{\text{IR}} \) channel (Ransom and Sontheimer 1995). Perfusion with Ba\(^{2+}\) for 70–90 min did not result in the induction of Cs-FP–like potentials (\( n = 4, \text{Fig. 7B} \)). This finding indicated that blockade of \( K_{\text{IR}} \) cannot explain the appearance of Cs-FPs. To investigate whether the induction of the Cs-FP depended on a simultaneous blockade of \( I_{\text{h}} \) and \( K_{\text{IR}} \), we co-applied ZD7288 and Ba\(^{2+}\), but observed no effect within 30–90 min of perfusion (\( n = 3 \)).

Another possible mechanism for the induction of the Cs-FP is a change in the activity of the Na\(^+\)/K\(^+\)-ATPase. It is known that Cs\(^+\) can be transported by the Na\(^+\)/K\(^+\)-ATPase in place of K\(^+\) (Aker et al. 1979; Sachs 1977). Therefore the presence of Cs\(^+\) might lead to a reduced transport of K\(^+\), and, as a result, extracellular K\(^+\) accumulation. Indeed, reduction of the Na\(^+\)/K\(^+\)
K⁺-ATPase activity by 20 μM dihydroouabain has been shown to provoke robust epileptiform burst activity in CA1 (Vailland et al. 2002). However, we found that dihydroouabain (20–40 μM, n = 2) failed to induce Cs-FP–like potentials. Not even co-application of Ba²⁺ (0.2 mM), ZD7288 (20 μM), and dihydroouabain (20 μM) could provoke the appearance of a Cs-FP (n = 1, data not shown).

4-AP is known to be an antagonist of several types of voltage-dependent K⁺ currents (Storm 1990) as well as the slow Ca²⁺-dependent K⁺ current (Andreasen 2002), and it has been shown to increase transmitter release (Barish et al. 1996; Kumamoto and Kuba 1985). 4-AP is also well-established as an epileptogenic agent in vitro (Perreault and Avoli 1992) and in vivo (Pena and Tapia 2000). In our experiments, addition of 4-AP (50 μM), after the block of ionotropic glutamate and GABA receptors, resulted in the development of a small positive field potential in three of seven slices. By increasing the concentration of 4-AP to 0.5 mM, we obtained a positive field potential in four of nine slices, with an average amplitude of 5.3 ± 1.4 mV and a duration of 99.9 ± 8.1 ms (Fig. 7D). When the stimulation intensity was increased from the range normally used (0.15–0.4 mA) to 0.6 mA, a field potential could be evoked in all slices tested (n = 8). These 4-AP–induced field potentials were somewhat similar to the initial phase of the Cs-FP. The negative component was, however, absent in six of eight experiments with 4-AP. A further increase in the concentration of 4-AP to 2 mM had no additional effect. These results show that 4-AP (0.5–2 mM) can, to some extent, mimic the actions of Cs⁺. Particularly striking, however, is the absence of a negative component in most of the experiments with 4-AP and the high stimulation intensity necessary.

Cs-FP is modulated by metabotropic glutamate receptor activation

Since our data indicated that the Cs-FP depended on synaptic transmission via receptors other than the ionotropic glutamate or GABA receptors, the next step was to examine the possible involvement of metabotropic glutamate receptors (mGluRs). Indeed, several subtypes of mGluRs have been shown to influence epileptic activity both in vitro and in vivo (Doherty and Dingledine 2002; Mordrich et al. 2003).

We found that application of the group I agonist DHPG (40 μM) (Wisniewski and Car 2002) significantly reduced the peak amplitude of the positive component (by 31.6 ± 10.4%, P = 0.04, n = 5) and increased its duration by 68.2 ± 13.9% (P < 0.01, Fig. 8A). The effect of DHPG was clearly dose-dependent in the range of concentrations between 40 and 400 μM (Fig. 9A). With 20 μM DHPG, the average response was larger than that seen with 40 μM. However, the effect of 20 μM DHPG was found to be statistically insignificant (P = 0.07) in contrast to the effect of higher concentrations.

The group I antagonist (5)-MCPG (Conn and Pin 1997) had no effect in itself when applied in a concentration of 1 mM (n = 4, results not shown). The racemic mixture (RS)-MCPG (1 mM) had no effect either and failed to antagonize the effect of 40 μM DHPG (n = 6, Figs. 8B and 9B). Because agonist-dependent antagonism of MCPG on mGluR5a has been described (Doherty et al. 1999), and since mGluR5a is expressed in the rat hippocampus (Shigemoto et al. 1997), we also tested the selective mGluR5a antagonist MPEP (Gasparini et al. 1999). As seen with MCPG, MPEP (10 μM) had no effect on the Cs-FP and did not antagonize the effect of 40 μM DHPG (n = 6, Figs. 8C and 9B). Taken together, these results indicate that neither the Cs-FP nor the inhibitory effect of DHPG is likely to be mediated through activation of group I mGluRs. Another group I agonist, QUIS (Conn and Pin 1997), had a similar effect on the Cs-FP as DHPG. Thus QUIS (0.1 mM) reduced the peak amplitude of the initial component by 67.6 ± 10.1% (P = 0.04, n = 3, Fig. 10C), an effect that corresponded to that obtained by 0.2 mM DHPG. Also, the effect of QUIS was not antagonized by 1 mM (5)-MCPG (data not shown).

The nonselective mGluR antagonist LY341495 (20 μM) (Kingston et al. 1998) had no effect on the Cs-FP (n = 6). However, when the concentration was increased to 0.1 mM, we found a significant reduction of the duration of the initial component, by 21.4 ± 6.2% (P < 0.01, n = 11), but no change

**FIG. 8.** Effect of (RS)-3,5-dihydroxyphenylglycine (DHPG). A: Cs-FP evoked before (control) and 15 min after addition of the mGluR agonist DHPG (40 μM). B and C: Cs-FP evoked before (control) and during perfusion with the group I mGluR antagonist (RS)-α-methyl-4-carboxyphenylglycine (MCPG) (B) or the selective mGluR5a antagonist 2-methyl-6-(phenylethynyl)pyridine hydrochloride (MPEP) (C). Right traces were recorded 25 min after co-application of DHPG. Note the lack of effect of either antagonist both on the field potential and on the inhibitory effect of DHPG.
in the amplitude (Fig. 10A). The effect of LY341495 also included an increase in the amplitude of the negative component. We also found that LY341495 (20 –100 μM) gave a partial reversal of the inhibitory effect of both DHPG (P = 0.04, n = 11) and QUIS (n = 2) on the Cs-FP (Fig. 10, B and C). To test for the possible involvement of mGluRs in the induction of Cs-FP, LY341495 (0.1 mM) was included during the wash-in of Cs⁺. In four of four experiments, typical Cs-FPs were fully developed within 60 min of perfusion, indicating that mGluRs are not involved in the induction process.

In an attempt to pinpoint more exactly the type of receptor involved, we tested the effects of two other ligands of mGluRs. The mGluR4a/mGluR8 antagonist MAP4 (0.5 mM) (Conn and Pin 1997; Saugstad et al. 1997) had no effect on the Cs-FP (n = 3). The group III agonist L-AP4 (0.2 mM) (Conn and Pin 1997) significantly increased the duration of the initial component, by 64.7 ± 22.8% (P = 0.02, n = 3), and at the same time, reduced its peak amplitude by 19.2 ± 10.8%. The latter effect, however, was not significant (P = 0.23). No additional effect was seen by increasing the concentration of L-AP4 to 1 mM.

**DISCUSSION**

**Cs-FP**

We have shown here that Cs⁺ promotes the development of a complex field potential in response to orthodromic stimulation of the Schaffer collateral-commisural fibers. This Cs-FP was relatively short in duration (145 ms) and was associated with synchronous discharge. In these two respects it resembles interictal activity recorded in other in vitro models of epileptogenesis. This indicates that the mechanisms underlying the Cs-FP could be involved in epileptiform activity in the hippocampus.

A long line of evidence indicates that the Cs-FP is synaptic in origin and caused by stimulation of the Schaffer collateral-commisural fibers impinging on the CA1 pyramidal cells. First, the Cs-FP is clearly Ca²⁺-dependent. Thus removal of extracellular Ca²⁺ or unspecific block of high-voltage–activated Ca²⁺ channels abolished the response. Much of the latter effect could be attributed to a block of N-type Ca²⁺ channels as judged from the marked effect of ω-conotoxin GVIA on the Cs-FP. Presynaptic N-type channels, together with P/Q-type Ca²⁺ channels, are now well established as providing the major routes for the Ca²⁺-influx which triggers glutamate release from Schaffer collateral-commisural fibers (Horne and...
not seen. However, we also found that Cs+ on the Cs-FP similar to that of adenosine. Such an effect was likely because Cs+ blocks the GIRK channels (Yamada et al. 1998), which would leave adenosine ineffective. The most likely explanation for these results is therefore a presynaptic action of adenosine. Also in agreement with a synaptic origin is the similarity between the Cs-FP and the field potential induced by 4-AP.

The spatial profile of the initial component (Fig. 4) has a similar distribution of positivity and negativity as a glutamatergic EPSP evoked by stimulation of the Schaffer collateral-commisural fibers (Andersen et al. 1966). Nevertheless, our results strongly suggest that neither the NMDA nor the non-NMDA glutamate receptors are involved, since the Cs-FP was unaffected by high concentrations of selective antagonists toward these receptors. Furthermore, the NMDA-channel blocker MK-801 that binds noncompetitively to the channel (Foster and Wong 1987) also had no effect on the Cs-FP, which rules out the possibility that Cs+ acted through interference with the binding of APV to the receptor. The Cs-FP was also largely unaffected by a number of antagonists toward mGluRs, in particular the nonselective antagonist LY341495 (0.1 mM), applied in a concentration sufficient to affect all known mGluRs (Kingston et al. 1998). On the basis of these observations, it is difficult to point to any simple mechanism by which glutamate could mediate the response, and this matter remains, at the present stage, open to speculation. A general enhancement of synaptic release (of glutamate and other transmitters) seems to be a necessary precondition for the development of this new type of synaptic event. There is some evidence for the existence of a CNQX- and APV-resistant glutamate receptor other than the metabotropic receptors (Mudrick and Heinemann 1990). The activation of this receptor gives rise to a field potential and induces changes in extracellular ions similar to those observed during non-NMDA receptor activation. In regard to the Cs-FP, further experiments are clearly needed to identify the receptor(s) and transmitter(s) involved. It should also be noted that the Cs-FP is not reminiscent of the previously reported Cs+-induced spontaneous epileptic activity, since the latter is abolished by antagonists of inotropic glutamate receptors (Hwa and Avoli 1991; Xiong and Stringer 1999).

As outlined earlier, we expected baclofen to have an action on the Cs-FP similar to that of adenosine. Such an effect was not seen. However, we also found that Cs+ reversed the inhibitory effect of baclofen on the normal fEPSP. This latter effect is known to be primarily presynaptic (Dumas and Foster 1998; Wu and Saggau 1997; Yoon and Rothman 1991). Therefore the most simple explanation for the lack of effect of baclofen on the Cs-FP is that Cs+ blocks the presynaptic effect of baclofen. The mechanism for this blockage is not immediately clear, since the presynaptic action of baclofen, rather than being mediated by GIRK channels, is currently believed to be coupled to N- and P/Q-type Ca2+ channels (Bowery et al. 2002; Wu and Saggau 1997).

Possible nonsynaptic mechanisms behind the Cs-FP

Because of the small extracellular space between CA1 pyramidal cells and their common orientation, they are susceptible to nonsynaptic excitation (Dudek et al. 1998; Jefferys 1995). The Cs-FP could possibly arise from a direct excitation of subpopulations of neurons near the stimulation electrode that, through electrical field interactions or leakage of (most likely K+) ions into the extracellular space, could have excited neighboring neurons. The all-or-nothing manner, in which the Cs-FP depends on stimulus intensity, is in favor of this hypothesis. Stretching the imagination, it is possible that postsynaptic N-type Ca2+ channels could be necessary for this electrical field interaction and hence provide an explanation for the effect of o-conotoxin GVIA, but the inhibitory effect of adenosine still remains to be explained, since the postsynaptic effects of adenosine are linked to GIRK-channels and not Ca2+ channels (Takigawa and Alzheimer 1999).

Electrical field interactions, ephaptic interactions, and fluctuations in ion concentrations are markedly less effective in altering neuronal excitability if the extracellular volume is expanded (Jefferys 1995; Schwartzkroin et al. 1998). By increasing the extracellular osmolarity with 30 mM sucrose, we would expect the Cs-FP to be dramatically attenuated, were it dependent of nonsynaptic mechanisms. However, we only observed a reduction equal to that of a normal orthodromic synaptic field potential. All in all, our results are not in support of a nonsynaptic mechanism underlying the Cs-FP.

Cs+ works through an unknown action

Cs+ is known to have a number of different actions, each of which were examined here. Our finding, that ZD7288 did not mimic the effect of Cs+ is in line with previous observations that this compound does not induce epileptiform activity (Gasparini and DiFrancesco 1997; Janigro et al. 1997; Xiong and Stringer 1999) and indicates that blockade of I\textsubscript{h} is insufficient for the induction of the Cs-FP.

Blockade of K\textsubscript{IR} channels would reduce glial K+ buffering and lead to extracellular K+ accumulation, which has been suggested to underlie Cs+-induced neuronal synchronization (Janigro et al. 1997). There is, however, some debate as to the validity of this hypothesis. First Cs+ blocks the I\textsubscript{ Kir} in a voltage-dependent manner, with little effect observed at resting membrane potential (Ransom and Sontheimer 1995). Second, Xiong and Stringer (1999) found that Cs+ did not alter [K+]o or the rate of [K+]o clearance after field bursts and that Cs+ caused burst activity in cultured hippocampal neurons, which have a larger and more unrestricted extracellular space than their in situ counterparts. In our experiments, application of Ba2+, which, at the concentration used here also blocks I\textsubscript{Kir} (Ransom and Sontheimer 1995), did not induce field potentials like the Cs-FPs. In the presence of baclofen alone, Ba2+ did induce an epileptiform potential (Fig. 6B). This potential differed, however, from the Cs-FP in two important ways: it had no negative component and could be completely blocked by CNQX, APV, and bicuculline. Thus blockade of I\textsubscript{Kir} does not seem a likely mechanism for the induction of the Cs-FP. The
possibility that Cs⁺ exerted its effect through an interference with the Na⁺/K⁺ pump (Akerja et al. 1979; Sachs 1977) is not supported by our data either, since a reduction of the activity of the Na⁺/K⁺ pump by dihydroouabain was unable to mimic the action of Cs⁺.

Cs⁺ has been reported to increase transmitter release in bullfrog sympathetic ganglia (Kumamoto and Kuba 1985) and in CA1 (Manabe et al. 1993). In our experiments, the ability of Cs⁺ to induce a CNQX- and APV-resistant field potential could be mimicked by 4-AP, which has also been shown to increase transmitter release in the hippocampus (Barish et al. 1996). This could indicate that the effect of Cs⁺ is, at least partly, dependent on an enhanced transmitter release, although the 4-AP-induced potential was not exactly identical to the Cs-FP. Furthermore, the long delay (40–70 min) for the development of the Cs-FP is in good agreement with the reported latency of about 50 min for the Cs⁺-induced enhancement of transmitter release (Kumamoto and Kuba 1985). Based on circumstantial evidence, we propose that a Cs⁺-induced increase in transmitter release is a prerequisite for the Cs-FP. It should be noted that, in these experiments, we have focused on the induction mechanism for the Cs-FP. It cannot be excluded, however, that changes in intrinsic membrane properties and/or extracellular [K⁺] are somehow involved in the response. Indeed, the prolonged synchronous firing associated with the stimulation and the very steep input-output curve for the Cs-FP are both indicative of a general enhancement of membrane excitability, presumably in the pyramidal cells. To resolve which parts of the response are due to the primary synaptic event and which are related to altered intrinsic properties, more detailed studies at the single cell level will be needed.

Metabotropic glutamate receptors modulate the Cs-FP

Because of the potential role of mGluR ligands as antiepileptic drugs, it is important to clarify how specific subtypes of this complex group of receptors can influence epileptiform activity. Although our data do not support a direct involvement of mGluRs in the maintenance or induction of Cs-FP, the response was clearly attenuated by the mGluR agonists DHPG and QUIS. Generally, receptors activated by both DHPG and QUIS are considered to belong to the group I mGluRs, members of which are reported to affect CA1 pyramidal cell excitability and synaptic transmission (Gereau and Conn 1995; Mannaioni et al. 2001). However, activation of group I mGluRs does not seem to be a likely explanation for the observed effects. First, the effective dose of DHPG is much higher than the EC50 value of DHPG on group I mGluRs, which is 6.6 μM on mGluR1a and 2 μM on mGluR8a (Wisniewski and Car 2002), and second, the antagonists MCPG (mGluR1a) and MPEP (mGluR8a) did not antagonize the effect of DHPG or QUIS. DHPG has also been reported to inhibit binding of (3H)-LY341495 to mGluR3 (K: 106 μM), but so has (RS)-MCPG (K: 165 μM) (Johnson et al. 1999). This means that the concentration of (RS)-MCPG used in our experiments (1 mM) should have inhibited the response to 40 μM of DHPG, had the DHPG effect been via mGluR3. For these reasons, it does not seem likely that mGluR3 receptors are responsible for the effect of DHPG and QUIS.

Group III mGluRs are believed to be located presynaptically, and mGluR7a has been localized to terminals of the Schaffer collateral-commisural fibers (Shigemoto et al. 1997). In our experiments, the effect of L-AP4, a group III agonist (Conn and Pin 1997), was similar to that of DHPG and QUIS, but L-AP4 was much less effective. Although QUIS seems to have some activity at group III mGluRs (Conn and Pin 1997), L-AP4 is reported to be much more potent. Therefore it seems rather unlikely that the effect of DHPG and QUIS is mediated through a group III mGluR. In another study, both L-AP4 and DHPG were shown to depress excitatory transmission in the Schaffer collateral-CA1 connection (Gereau and Conn 1995), which would be in agreement with our observations. However, in contrast to our findings, Gereau and Conn (1995) found that the DHPG effect was reversed by (S)-MCPG (0.5 mM). As a final possibility, the effect of DHPG could be exerted via the phospholipase D–coupled mGluR, which is of a distinct type that does not fit the pharmacological profile of any other mGluR (Boss et al. 1994; Pellegrini-Giumpietro et al. 1996). DHPG in high concentrations has been reported to antagonize the formation of phospholipase D, whereas QUIS acts as a highly potent agonist (Pellegrini-Giumpietro et al. 1996). Since we have observed similar effects of DHPG and QUIS on the Cs-FP, it seems unlikely that this receptor is involved in the modulation of the Cs-FP to any great extent. All in all, the pharmacological profile of the receptor through which DHPG and QUIS reduce the Cs-FP does not fit that of any of the currently known mGluR.

We observed two different effects of LY341495—a reversal of the effect of DHPG and QUIS and a shortening of the positive component. The former effect suggests that LY341495 is an antagonist on the receptor that has DHPG and QUIS as agonists. The latter effect could be mediated by any of a number of receptors, because LY341495 is an antagonist of group I, II, and III mGluRs (Kingston et al. 1998). However, the involvement of group II mGluRs is unlikely because nanomolar concentrations of LY341495 are effective at this group (Kingston et al. 1998). Furthermore, the lack of effect of MCPG, MAP4, and MPEP rules out mGluR1a, mGluR5a, mGluR8a, and mGluR9a (Conn and Pin 1997; Gasparini et al. 1999; Saugstad et al. 1997). mGluR8a is not believed to be expressed in the hippocampus (Shigemoto et al. 1997). Therefore the most likely receptor for this effect is mGluR7a. However, the IC50 of LY341495 toward this receptor is very low (0.99 μM) (Kingston et al. 1998) compared with the high concentration needed in this study.

In conclusion, our study has shown that excitatory synaptic transmission can exist between area CA3 and area CA1 via the Schaffer collateral-commisural fibers independently of the known ionotropic and metabotropic glutamate receptors. This type of excitatory transmission seems to be mediated via a hitherto undescribed mechanism, which would be selectively expressed under circumstances where synaptic transmission in general is enhanced and which is subject to modulation via mGluR activation. The resulting epileptiform potential differs fundamentally in its mechanism from other types of epileptiform activity. Future studies should be aimed at a precise identification of the transmitter system(s) and receptor(s) involved and provide a more detailed account of the necessary conditions for their activation.

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Miles R and Wong RKS. Excitatory synaptic interactions between CA3 pyramidal neurons in the guinea pig hippocampus. J Physiol 373: 397–418, 1986.


