Heterogeneous firing behavior during ictal-like epileptiform activity in vitro

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It is well established that extracellular Cs⁺ (3–5 mM) can induce spontaneous epileptiform activity in neocortical or hippocampal brain slices that depends on both synaptic and nonsynaptic elements (D’Ambrosio et al. 1998; Hwa and Avoli 1991; Janigro et al. 1997; Kraglund et al. 2010; Xiong and Stringer 1999). In addition, long-term application of Cs⁺ promotes a stimulus-induced epileptogenic field potential (Cs-FP) in area CA1. This potential is elicited by stimulation of the Schaffer collaterals and consists of a positive-going phase, lasting on average 145 ms, followed by a negative phase, which lasts several seconds. Evidence suggests that the positive phase depends on a synaptic trigger (which is facilitated by Cs⁺), or alternatively by 4-aminopyridine, and exists in the presence of high concentrations of ionotropic glutamate and GABA receptor antagonists, the nature of which has not yet been identified (Skov et al. 2005). However, recent data from an ongoing investigation suggest that the Cs-FP can be blocked in the combined presence of two different AMPA receptor antagonists, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and GYKI52466 in high doses (Andreasen and Nedergaard, unpublished observations), thus compatible with the involvement of fast glutamatergic transmission at the CA3–CA1 synapse. The duration of the negative phase is typically 2–4 s but can exceed 10 s in some slices and is usually accompanied by synchronized population spike activity. The shape and temporal relation between the two phases have a close similarity to the spontaneous interictal-ictal events induced by raised extracellular K⁺ concentration ([K⁺]o) (Jensen and Yaari 1988; Trayanil and Dingledine 1988), indicating that the positive and negative phases are similar in nature to interictal and ictal activity, respectively. There are several aspects of the Cs-FP that make it a relevant model for studying the dynamics of seizure-like events. First, the epileptiform activity seems to depend on a complex interaction between synaptic and nonsynaptic elements in addition to changes in neuronal properties (Andreasen et al. 2007; Skov et al. 2005, 2009). There is now much evidence for a similar complexity involved in icetogenesis in human epileptic tissue (Avoli et al. 2005; Bragin et al. 2000; McNamara et al. 2006; Scharfman 2007; Traub et al. 1996). Second, the progression of the Cs-FP depends on the blockade of glial inward-rectifying K⁺ (Kᵢg) channels (Andreasen et al. 2007). A downregulation of glial Kᵢg channels is frequently encountered in pathological conditions such as temporal lobe epilepsy (Bordey and Sontheimer 1998; D’Ambrosio 2004; Hinterkeuser et al. 2000; Steinhäuser and Seifert 2002). Third, in contrast to several other types of in vitro models (reviewed in Jefferys 1995), the Cs-FP is only partly dependent on extracellular ion movements and field effects (Andreasen et al. 2007). The importance of such nonsynaptic interactions in temporal lobe seizures has been questioned, as pyramidal cells in human hippocampi are less
closely packed compared with rodent hippocampi (reviewed in McCormick and Contreras 2001).

Our previous studies have shown that the response of pyramidal cells during the initial (interictal-like) phase of the Cs-FP shows little variability and consists of high-frequency burst firing time-locked to population spikes (Skov et al. 2009). In contrast, preliminary observations have indicated a high diversity of response patterns among neurons, including absence of discharge, during the ictal-like phase. The response patterns across a population of neurons during ictal events are not well described in vitro. However, several in vivo studies have shown a highly heterogeneous population behavior (Matsunoto and Marsan 1964; Steriade et al. 1998; Wyler et al. 1975, 1978; Wyler and Ward 1986), the distribution of which is found to be correlated with the severity of the seizure in patients with temporal lobe epilepsy (Babb et al. 1987). The mechanisms for such diversity remain unknown.

Since the population behavior during stimulus-induced ictal-like events, in the presence of Cs\(^+\), may thus imitate the heterogeneous population behavior observed during in vivo seizure activity, a study of the dynamics of individual neurons could potentially provide new insight into the control of network behavior during seizures. The aim of this study was therefore to characterize and subdivide the population of CA1 pyramidal neurons in terms of individual firing behavior during epileptiform activity, and to investigate the possible causes, including intrinsic ionic currents, that could explain the diversity of response patterns.

MATERIALS AND METHODS

Animal care and housing facilities as well as the experimental protocol for the euthanasia of animals were in accordance with Danish and European law and approved by the Animal Experimentation Board under the Danish Ministry of Justice. Experiments were performed on hippocampal slices prepared from male Wistar rats (4–5 wk old). Rats were anesthetized with isoflurane and decapitated. The protocols for the euthanasia of animals were in accordance with Danish legislation (gift from Dr. Palle Christoffersen (Neurosearch, Glostrup, Denmark)).

Conventional recording techniques were employed, using a high-input impedance amplifier (Axoclamp 2A, Molecular Devices) with bridge balance and current injection facilities. Signals were digitized online via a Digidata 1440 interface and transferred to a computer for analysis employing pCLAMP (version 10, Molecular Devices).

Slices were accepted if they, under control conditions, displayed a normal orthodromic field potential with a single population spike (peak amplitude: <15 mV, defined as the maximal response) and no evidence of secondary population spikes with supramaximal stimulation. Intracellular recordings were accepted if the cells had resting membrane potentials (RMP) < −55 mV, input resistance (R\(_{\text{in}}\)) > 15 M\(\Omega\), and action potential (AP) height > 80 mV.

Analysis. The transmembrane potential was measured as the voltage difference between the intra- and extracellular recording electrodes. R\(_{\text{in}}\) was measured as the slope of the current/voltage (IV) curve close to RMP; the AP threshold was estimated with short (4 ms) depolarizing current pulses of increasing intensity. The temporal relationship between APs and population spikes was evaluated by measuring the time interval (Δt = t\(_{\text{AP}}\) − t\(_{\text{pop.spike}}\)) between the peak of the population spike and the peak of the nearest AP (see Fig. 7A). The area of the afterdepolarization (ADP; see inset in Fig. 8C) following each AP was measured between the peak of the fast afterhyperpolarization and the point at which the ADP had decayed back to baseline (Yue and Yaari 2004). The epileptiform field potential induced in the presence of Cs\(^+\) (Cs-FP) was quantified by measuring the area of its two components. The area of the positive (interictal-like) phase was measured from the onset of the response to the point where the potential reached the prestimulus baseline, and the area of the negative (ictal-like) phase was measured from the end of the positive phase to the end of the Cs-FP. The peak amplitude of the positive phase was measured with respect to the prestimulus baseline potential, and its duration was measured from onset to the point where it reached the prestimulus baseline. All measurements were done on the average of 3–10 responses.

Values are given as means ± SE unless otherwise indicated. For statistical evaluation the paired or unpaired Student’s t-test, the Mann-Whitney U-test, and the one-way ANOVA test were used as appropriate, with a level of significance set at 5%. Drugs and solutions. The composition of the dissection medium was (in mM) 120 NaCl, 2 KCl, 1.25 KH\(_2\)PO\(_4\), 6.6 HEPES acid, 2.6 NaHEPES, 20 NaHCO\(_3\), 2 CaCl\(_2\), 2 MgSO\(_4\), and 10 D-glucose, bubbled with carbogen. The composition of the standard perfusion medium was (in mM) 124 NaCl, 3.25 KCl, 1.25 NaH\(_2\)PO\(_4\), 20 NaHCO\(_3\), 2 CaCl\(_2\), 2 MgSO\(_4\), and 10 D-glucose, bubbled with carbogen (pH 7.3).

To induce epileptiform activity, Cs\(^+\) (5 mM) was added to the standard perfusion medium together with ionotropic glutamatergic and GABAergic receptor antagonists [CNQX (10 μM), (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclo-hepten-5,10-imine maleate (MK801, 10 μM), and bicuculline methobromide (BIC, 1 μM)].

Most pharmacological compounds were made up in aqueous stock solutions of 100–1,000 times the required final concentration and diluted in the perfusion medium as appropriate. Linopirdine and retigabine were dissolved in ethanol and DMSO, respectively. The lidocaine derivative QX314 was dissolved in 4 M K\(^+\) acetate.

CNQX, MK801, BIC, linopirdine, phenytoin, QX314, and tetrodotoxin (TTX) were purchased from Sigma. Retigabine was a kind gift from Dr. Palle Christoffersen (Neurosearch, Glostrup, Denmark).

RESULTS

Cs\(^+\) -induced changes in basic membrane properties of CA1 pyramidal neurons. In accordance with previous findings (Andreasen et al. 2007), the addition of Cs\(^+\) (5 mM) together with the antagonists (see MATERIALS AND METHODS) resulted, over a period of
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Fig. 1. Cs\(^{+}\)-induced changes in the basic membrane properties of CA1 pyramidal neurons. A: extracellular recordings showing the temporal development of a stimulus-induced epileptiform field potential (Cs-FP) in the presence of Cs\(^{+}\) (5 mM), CNQX (10 \(\mu\)M), MK801 (10 \(\mu\)M), and bicuculline (10 \(\mu\)M). In this and subsequent figures arrowheads mark the time of orthodromic stimulation. B: plots of changes in input resistance (\(R_{in}\)), membrane potential (\(V_m\)), and action potential (AP) threshold of a CA1 pyramidal neuron recorded during development of the Cs-FP shown in A. In this and subsequent figures, the intracellular and extracellular recording sites were obtained in close proximity. C: histograms of average values of \(R_{in}\), \(V_m\), and AP threshold in relationship to time (20, 40, and 60 min; C, control) in Cs\(^{+}\). Numbers in parentheses denote \(n\). *P < 0.05.

~60–70 min, in the development of the Cs-FP (Fig. 1A; see Andreasen et al. 2007 for detailed description of Cs-FP and possible mechanism of action of Cs\(^{+}\)). During washin of Cs\(^{+}\), individual neurons, recorded in the absence of synaptic stimulation, depolarized with a concomitant increase in \(R_{in}\) and, frequently, also in firing threshold (Fig. 1B). After 60-min perfusion of Cs\(^{+}\), the average RMP was depolarized by 5.6 mV and \(R_{in}\) was increased by 8.6 M\(\Omega\). These changes reached statistical significance (\(P < 0.05\)) after 20- and 40-min perfusion, respectively (Fig. 1C). On average, the firing threshold reached a stable level (~2.7 mV above control level) after 20 min that was not significant (\(P \approx 0.056\)).

Heterogeneous population behavior during Cs\(^{+}\)-induced epileptiform activity. Next, we monitored the behavior of single neurons during the fully developed stimulus-induced Cs-FP. The transition between the positive and negative parts is characterized by a shift in the polarity of the tonic field potential and a transient decrease in the amplitude of synchronized population activity (Fig. 2A). The majority (90%) of neurons responded with prolonged firing extending through both phases (Fig. 2B), whereas 10% only generated a short (~100 ms) high-frequency burst (Fig. 2C). Henceforth, these two populations of neurons will be referred to as “active” and “passive,” respectively. The construct of the short bursts observed in “passive” neurons (Fig. 2C) resembles that of a paroxysmal depolarizing shift (PDS) underlying interictal activity (reviewed in de Curtis and Avanzini 2001; McCormick and Contreras 2001). In support of an equivalence to PDS, our earlier results showed that the initial event depends on a synaptic trigger (Skov et al. 2005). APs evoked during the PDS-like burst were time-locked to the highly synchronous population activity observed during the upstroke of the positive phase of the Cs-FP. Burst activity in “active” neurons began with a short period of high-frequency firing with characteristics similar to the PDS-like burst observed in “passive” neurons (compare Fig. 2, B and C). This suggests that the initial

Fig. 2. Configuration of Cs\(^{+}\)-induced epileptiform activity. A: extracellular (top) and intracellular (bottom) recordings of an evoked epileptiform response. The field response (Cs-FP) is composed of an initial positive (interictal-like) phase followed by a prolonged negative (ictal-like) phase. Note that the transition between the 2 phases is characterized by a decrease in the amplitude of population spikes. The CA1 pyramidal cell responds with an initial high-frequency burst that continues as prolonged afterdischarge. B: enlarged view of traces in A. During the upstroke of the positive phase, APs are time-locked to the synchronous population activity. Concurrently with the decreased synchrony of population activity during the transition period, the frequency of APs decreases to about half that of the population spikes. After the transition period the population activity becomes highly synchronized and the APs are time-locked to the population spikes (A). C: “passive” pyramidal neurons only generate a short high-frequency burst corresponding to the upstroke of the Cs-FP. This isolated burst is defined as the primary burst, and subsequent firing, if any, is defined as afterdischarges (B). Resting membrane potential (RMP): ~68 mV (A), ~63.8 mV (C).
PDS-like response constitutes a primary burst (Borck and Jefferys 1999; de Curtis and Avanzini 2001; Jensen and Yaari 1997; Traub et al. 1996) that, through synchronization across the population, initiates events that lead to prolonged afterdischarges in “active” neurons.

To elucidate the mechanisms responsible for the afterdischarge of “active” neurons, we hyperpolarized the membrane during the primary burst by injection of a square current pulse (−0.3 nA), lasting from 50 ms before to 100 ms after the afferent stimulus (see Fig. 3). This procedure introduced a delay between the primary burst and the afterdischarge, allowing the latter to be observed in isolation. Analyses of a large population of neurons showed distinct patterns of afterdischarge, which could be categorized into three main subtypes (I–III). In neurons with type I behavior, the primary burst was followed by a depolarizing plateau potential of variable length characterized by a fast upstroke and slow decay. High-frequency firing (31.2 ± 2.4 Hz, n = 30) was observed on top of the plateau (Fig. 3A). Stepwise steady hyperpolarization of the membrane gave a progressive increase in the latency between the primary burst and afterdischarges. The afterdischarge vanished along with the plateau in an all-or-none manner at a threshold of −58.6 ± 0.9 mV (n = 21). Below threshold, an additional, low-amplitude, depolarization was revealed (see

Fig. 3. Heterogeneous population behavior during Cs⁺-induced ictal-like activity. Afterdischarge patterns of pyramidal neurons with type I (A), type II (B), and type III (C) behavior in relationship to simultaneously recorded Cs-FPs (extracellular trace). A: type I behavior recorded at 4 different holding potentials (indicated on left), demonstrating the voltage-dependent plateau potential. A hyperpolarizing current pulse (−0.3 nA, 150 ms) is injected during the initial part of the response. Note persistence of the primary burst during hyperpolarization. Bottom: superimposition of the responses evoked at −73 mV (black) and −75 mV (red) emphasizing the slow, voltage-independent depolarization. B: type II behavior consisting of low-frequency random afterdischarge. Steady depolarization reveals a small hyperpolarization following the primary burst. C: type III behavior is characterized by high-frequency afterdischarge riding on top of a slow voltage-independent depolarization. The afterdischarge is time-locked to the population spikes. An enlarged view of the base of the AP marked by * is shown in inset to emphasize the hyperpolarizing prepotential (arrow) preceding each AP.
superimposition in Fig. 3A), which showed little voltage dependence even during large hyperpolarizations to near $-90 \text{ mV}$ (not shown) and had a duration that closely matched that of the negative phase of the Cs-FP. Henceforth, we refer to this component as the passive depolarization.

Neurons with type II behavior displayed a passive depolarization but no voltage-dependent plateau. The afterdischarge was superimposed on the passive depolarization and had a low frequency ($3.9 \pm 0.5 \text{ Hz}$, $n = 20$), which was often irregular (Fig. 3B). The overall firing rate increased in response to steady depolarization, but it never attained frequencies observed in neurons with type I behavior (compare Fig. 3, A and B). Furthermore, the primary burst was often followed by a silent period of several hundred milliseconds before onset of the afterdischarge (Fig. 3B). In the majority (72%) of experiments with type II behavior, there was little population spike activity during the negative phase of the Cs-FP. In the remaining experiments, population activity was, however, pronounced, suggesting that type II behavior is not linked to a lack of synchronized population activity.

Type III behavior was distinguished by high-frequency ($27.6 \pm 2.9 \text{ Hz}$, $n = 13$) afterdischarge in the absence of plateau potentials (Fig. 3C). A passive depolarization was present, but in contrast to type I and II behavior, each AP arose abruptly from a transient hyperpolarizing prepotential coinciding with a population spike (see inset, Fig. 3C). Note that the hyperpolarization is presumably caused by the action current induced by population spikes and therefore represents a depolarization of the transmembrane potential (see DISCUSSION). Plateau potentials could not be induced by steady depolarizing current injection.

In “passive” neurons afterdischarge was absent even during strong steady depolarizing current injection (Fig. 4A). At depolarizing holding potentials, the primary burst was often followed by a prolonged hyperpolarization that corresponded, in time, with the negative phase of the Cs-FP. Passive behavior was not associated with any noticeable deviation in size, shape, or activity pattern during the Cs-FP, giving no evidence that these neurons were generally located away from the area of epileptiform activity (Fig. 4A). As evident in Fig. 4, the action current induced by population spikes gave rise to small transient potential changes in “passive” neurons, also suggesting a close proximity.

Of the total sample of 71 neurons investigated, 31 (43.7%) displayed type I behavior, 20 (28.2%) displayed type II behavior, 13 (18.3%) displayed type III behavior, and 7 (9.9%) were “passive” (Fig. 4B). The behavioral phenotype of individual neurons was stable over time, even during prolonged recordings with multiple repetitions of the stimulus protocol. Table 1 shows the average RMP, $R_{\text{in}}$, and firing threshold for neurons penetrated in control conditions. There was no significant difference in these parameters between neurons adopting different behavioral phenotypes during Cs-FP. For neurons penetrated in the presence of Cs$^+$ and antagonists, the properties are shown separately in Table 2. In this condition, there was a significant difference ($P = 0.04$, ANOVA) in RMP, with type II and type III neurons being somewhat more depolarized. There was, however, no significant difference in $R_{\text{in}}$ and firing threshold.

Properties of type I behavior. To examine whether plateau potential expression depends on the timing from the primary burst, we varied the delay between the two events by changing the duration of the hyperpolarizing current pulse. In all experiments, the plateau was absent during the hyperpolarization but

<table>
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<tr>
<th>Type</th>
<th>Average RMP, $R_{\text{in}}$, Threshold, mV</th>
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<tr>
<td>Type I</td>
<td>$-68.4 \pm 1.3$, $33.7 \pm 2.2$</td>
</tr>
<tr>
<td>Type II</td>
<td>$-68.2 \pm 0.8$, $43.7 \pm 2.1$</td>
</tr>
<tr>
<td>Type III</td>
<td>$-69.2 \pm 1.3$, $35.8 \pm 3.5$</td>
</tr>
<tr>
<td>Passive</td>
<td>$-71.6 \pm 2.2$, $35.6 \pm 1.2$</td>
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Data are means ± SE. RMP, resting membrane potential; $R_{\text{in}}$, input resistance.
was triggered upon repolarization irrespective of the duration of the hyperpolarization (Fig. 5A). The amplitude of the delayed plateau and the frequency of the superimposed afterdischarge were unaltered compared with control. It is noteworthy that the termination of the afterdischarges in all cases occurred before or in concert with the decay of the Cs-FP (Fig. 5A). It is possible that afterdischarges were induced by anodal break excitation following the hyperpolarizing current pulse (Lacaille and Williams 1990), but this seems unlikely because the anodal break excitation generally was not pronounced, as observed when afferent stimulation failed to evoke a Cs-FP and no afterdischarge was induced (Fig. 5A).

In some neurons with type I behavior the afterdischarge terminated before the decay of the Cs-FP (see Fig. 3A), indicating that intrinsic neuronal factors could influence the length of afterdischarge. We therefore examined the effect of hyperpolarization during ongoing afterdischarge and found that a transient termination of the activity by hyperpolarizing current injection of variable intensity and duration did not alter the course of spontaneous afterdischarge or, in other ways, change the characteristics of ongoing burst activity (Fig. 5B).

Prolonged epileptiform burst firing similar to type I behavior has been attributed to the activation of a persistent noninactivating Na\(^+\) current (\(I_{\text{NaP}}\)) (reviewed in Bikson et al. 2002; Kager et al. 2007; Stafstrom 2007; Yaari and Beck 2002). Since the above results indicated that the plateau potential underlying type I behavior is caused by an intrinsic voltage-dependent current, we investigated the possible role of \(I_{\text{NaP}}\) in Cs- or TTX in low concentrations (0.2 \(\mu\)M) greatly reduced the negative phase of the Cs-FP (Fig. 6A, left). The area of the negative phase was significantly reduced (25 \(\pm\) 11% of control value; \(P < 0.05\), \(n = 6\)) after 26-min perfusion (Fig. 6B) and nearly blocked after 39 min (6 \(\pm\) 4% of control; \(n = 5\)). In the same period, the positive phase of the Cs-FP initially increased to 130 \(\pm\) 26% of the control value (13 min), followed by a progressive decrease to 55 \(\pm\) 24% (39 min; Fig. 6B). Neither of these changes reached statistical significance (\(P \geq 0.16\)). At low concentrations (0.5 \(\mu\)M), TTX has been shown to greatly suppress \(I_{\text{NaP}}\)-induced voltage changes before affecting the transient \(I_{\text{NaT}}\) underlying the AP (Azouz et al. 1996; Franschetti et al. 1995). This explains why the positive phase is not blocked in the presence of TTX even though this phase depends on presynaptic APs (Andreasen et al. 2007; Skov et al. 2005). In neurons with type I behavior (3/7), TTX blocked the plateau potential in parallel with the reduction of the negative phase of the Cs-FP without affecting the primary burst (Fig. 6A, right). To block the \(I_{\text{NaP}}\) without concomitant alteration of the population activity, we made intracellular recordings with electrodes containing low concentrations (12.5–25 mM) of QX314. In four experiments, we found that the plateau potential and afterdischarge were abolished shortly after impalement of the neuron (Fig. 6C). This occurred without any discernable change in the primary burst. In two experiments we passively depolarized the neuron after blockade of type I behavior. This treatment provoked afterdischarge typical of type III behavior.

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**Table 2. Basic membrane properties of cells penetrated in presence of Cs\(^+\)**

<table>
<thead>
<tr>
<th></th>
<th>Type I ((n = 10))</th>
<th>Type II ((n = 13))</th>
<th>Type III ((n = 8))</th>
<th>Passive ((n = 2))</th>
</tr>
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<tbody>
<tr>
<td>RMP, mV*</td>
<td>(-65.8 \pm 1.9)</td>
<td>(-60.3 \pm 1.3)</td>
<td>(-61.2 \pm 1.8)</td>
<td>(-69.0 \pm 0.9)</td>
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<tr>
<td>(R_m), (\Omega)</td>
<td>(41.8 \pm 3.1)</td>
<td>(46.9 \pm 5)</td>
<td>(38.4 \pm 2.7)</td>
<td>(55.3 \pm 22.5)</td>
</tr>
<tr>
<td>Threshold, mV</td>
<td>(-55.4 \pm 1.8)</td>
<td>(-54.5 \pm 1.4)</td>
<td>(-57.1 \pm 1.2)</td>
<td>(-52.3)†</td>
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Data are means \(\pm\) SE. *ANOVA test gave a significant difference in membrane potential (\(V_m\)) among the 4 groups (\(P = 0.04\)). †\(n = 1.\)

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**Fig. 5.** Type I behavior involves both intrinsic and extrinsic factors. A: type I afterdischarge recorded in the absence of current injection (intracellular, top panel) and with hyperpolarizing current injection (–0.3 nA, 2,400 and 4,800 ms in the 2 bottom panels). Simultaneously recorded Cs-FPs are shown superimposed (extracellular). An additional trace (red) is included, which shows a trial in which the Cs-FP displayed a low-amplitude negative phase (not shown). Note the lack of an anodal break excitation (arrow). B: records from another cell of a control response (black) and a response where ongoing afterdischarge is interrupted by a hyperpolarizing current pulse (–0.3 nA, 1.2 s, red). RMP: –62 mV (A), –65.9 mV (B).
absence of a plateau and presence of hyperpolarizing prepotentials preceding each AP (Fig. 6C). To supplement the above findings, we used the antiepileptic drug phenytoin, which has been shown to preferentially reduce \( I_{\text{NaP}} \) in CA1 pyramidal neurons with an \( IC_{50} \) of 34 \( \mu \)M (Chao and Alzheimer 1995; Segal and Douglas 1997). In accordance with the results with TTX, we found that 30-min perfusion with phenytoin (50 \( \mu \)M) gave a significant reduction of the negative phase of the Cs-FP (29 \( \pm \) 3\% of control value; \( P < 0.05 \), \( n = 6 \)) with a concomitant nonsignificant increase in the positive phase (128 \( \pm \) 22\% of control value; \( P > 0.05 \%), not shown).

**Temporal relationship between neuronal discharge and population activity.** The above finding, that neurons with type I and III behavior both displayed high-frequency burst firing, would seem to indicate that expression of a plateau had no direct bearing on the discharge. On the other hand, a hallmark of type III behavior is the presence of hyperpolarizing prepotentials indicating that such discharge largely depends on recruitment (see Discussion). We examined this more closely by measuring in these two cell types the time interval (\( \Delta t \); see Materials and Methods and Fig. 7A) between the peak of a population spike and the peak of the nearest AP. The analysis was performed during the negative phase (see Fig. 2A) to ensure that the measurements were done during a period of highly synchronous population firing (see Fig. 2A), making small separations between the extra- and intracellular electrodes less critical (Bikson et al. 2003). Data from nine neurons with type III behavior showed that the APs were clustered within 3–4 ms of the population spike peak (Fig. 7A; mean \( \Delta t: -0.97 \pm 1.04 \) ms; \( n = 171 \)). In 10 neurons with type I behavior, all APs generated during the first half of the plateau potentials were triggered from depolarizing prepotentials (see inset in Fig. 7B). Most of these APs were clustered before and close to the peak of the population spike (Fig. 7B, mean \( \Delta t: -1.57 \pm 4.70 \) ms; \( n = 216 \)), but a significant proportion were distributed at longer delays on either side. During the second half of the plateau, APs arising from a hyperpolarized prepotential were observed in four neurons with type I behavior. The differences in AP distribution between type I and type III neurons are shown in a cumulative fraction plot in Fig. 7C. Comparisons of the two data sets by Kolmogorov-Smirnov test (\( P < 0.001 \)) and Mann-Whitney U-test (\( P = 0.01 \), 2-sided test) both showed a significant difference. The average length of the negative slope region of the population spike, which is primarily determined by fast somatic current (Varona et al. 2000), was measured in 12 randomly selected experiments to be 3.6 \( \pm \) 1.2 ms (mean \( \pm \) SD; \( n = 100 \)). We found that 64\% of the APs in type I and 90\% of APs in type III neurons were confined to this region (Fig. 7C). On the basis of these results we conclude that the presence of the plateau potential yields high-frequency firing that is less tightly bound to the population activity than seen in the absence of a plateau.

**Intrinsic burst firing.** It is well established that CA1 pyramidal neurons can be divided into regular firing neurons and intrinsic burst firing neurons (“bursters”) based on their response to threshold stimulation (Jensen et al. 1994; Schwartzkroin 1975; Yue et al. 2005). Furthermore, the bursting property has been suggested to predispose for a role as pacemaker during epileptiform activity (Chagnac-Amitai and Connors 1989; Yaari and Beck 2002). We investigated whether there is a correlation between the intrinsic firing properties and the type of behavior adopted during an ictal-like event. Neurons were classified as regular firing or “bursters” according to the criteria used by others (Jensen et al. 1994; Yue et al. 2005; see inset in Fig. 8A). We refrained from further subclassification of “bursters” (Jensen et al. 1994). In control conditions, “bursters” constituted 29\% (6/21) of the population...
(Fig. 8A), which is a slightly larger proportion than that reported by others under similar conditions (Jensen et al. 1994; but see Azouz et al. 1994). Prolonged exposure to Cs⁺ only gave a marginal increase in the ratio of “bursters” to 33% (7/21). We next related the afterdischarge behavior to the pre-Cs⁺ intrinsic firing properties. As shown in Fig. 8B, “bursters” fell into three categories of afterdischarge behavior (I, II, and “passive”). The data gave no support for a strong association between intrinsic bursting properties and type I behavior, as the proportion of “bursters” that adopted type I behavior (33%) was not higher than regular firing neurons (60%). It is noteworthy that 50% of “bursters” became “passive,” whereas only 13% of regular firing neurons fell into this group.

Yue et al. (2005) have found a positive correlation between the size of the ADP following individual APs and the capability of intrinsic burst firing. In CA1 pyramidal neurons, the ADP, which results from the activation of I_{NaP} (Azouz et al. 1996; Yue et al. 2005), is under the strict control of voltage-gated K⁺ currents (Yue and Yaari 2004). Furthermore, the changes observed during epileptiform activity (e.g., a rise in [K⁺] and a decrease in [Ca²⁺]), are expected to enhance the ADP (Somjen and Müller 2000; Su et al. 2001) and thus increase the ratio of “bursters” (Jensen et al. 1994; Yue et al. 2005). As expected, we found no correlation between the type of afterdischarge adopted and the area of the pre-Cs⁺ ADP (Fig. 8C). However, we did observe a general increase in the size of the ADP in response to Cs⁺ (Fig. 8D), which was highly significant (P = 0.008) in neurons adopting type I behavior, amounting to ∼160% (from 225 ± 39 mV·ms to 588 ± 114 mV·ms; n = 11; Fig. 8E). In contrast, the ADP only increased ∼45% (from 263 ± 28 mV·ms to 380 ± 71 mV·ms; n = 10) in neurons adopting non-type I behavior (not significant). In neurons that adopted type I behavior, an additional AP was occasionally induced during the amplified ADP (Fig. 8D); this was not seen in non-type I neurons.

Expression of type I behavior is controlled by the M current. It has been suggested that the size of the ADP is determined by a balance between the M current (I_{M}) and I_{NaP}, and blocking I_{M} greatly increases the frequency of “bursters” (Yue and Yaari 2004; Yue et al. 2005). We therefore tested the effect of linopirdine (10 μM), a relatively selective I_{M} antagonist (Aiken et al. 1995; Schnee and Brown 1998). We incubated the slices with linopirdine for 30 min prior to the addition of Cs⁺ and monitored the response to threshold-straddling depolarization. The threshold response changed, in all neurons (n = 10), from a single AP to a short burst of APs, confirming the block of I_{M} (Aiken et al. 1995; Yue and Yaari 2004, 2006) (Fig. 9A). The response to Cs⁺ perfusion in slices preincubated with linopirdine (n = 10) exhibited two prominent features that were different from control conditions. First, the ratio of neurons expressing type I behavior was 100% (10/10 neurons recorded). Second, the latency period for full development of the Cs-FP (control: 60–70 min; Andreasen et al. 1995; Schnee and Brown 1998) was reduced in linopirdine to 20–30 min (Fig. 9B). This was manifested as a significantly larger negative phase after 30-min perfusion (P < 0.05; Fig. 9C). No significant difference was found, however, when comparing the average size of the fully developed response in the two conditions (P = 0.16). Adding linopirdine (10 μM) after 30-min perfusion of Cs⁺ had no significant effect on either the evolution or the size of the fully developed Cs-FP (P = 0.80, n = 11).

To further evaluate the possible regulatory function of I_{M}, we used retigabine, a selective KCNQ2/3 potassium channel opener (Main et al. 2000). Retigabine (50 μM) gave an overall reduction of the Cs-FP (Fig. 10A), involving a 76.4% decline in the area of the negative phase (P = 0.001, n = 9). The dimensions of the positive phase were also significantly reduced by retigabine (P < 0.03; Fig. 10). The effect of retigabine on the positive phase was fully reversible, whereas its effect on the negative phase was only slightly reversed after
DISCUSSION

Heterogeneous firing behavior during ictal-like activity. The present results show differential activity patterns among pyramidal neurons during epileptiform population activity, suggesting that an individual neuron will make different contributions to the ensemble activity of the network depending on the behavior it adopts. We found that the division into different behavioral patterns was manifested during the prolonged ictal-like phase of the Cs-FP. In contrast, the initial phase, which is reminiscent of the primary burst or PDS described in other models (reviewed in de Curtis and Avanzini 2001; McCormick and Contreras 2001), was associated with a homogeneous neuronal behavior. Similar to the PDS, the primary burst induced in Cs⁺ depends on synaptically driven activation of intrinsic conductances (Andreasen et al. 2007; Skov et al. 2005). Such direct activation is likely to yield a rather uniform response among neurons and may explain why the population in this phase is remarkably homogeneous. Development of the ictal-like phase depends on nonsynaptic interactions (Andreasen et al. 2007; Skov et al. 2005), and the accompanying neuronal discharge will therefore result from and/or contribute to such interactions. During this phase, 10% of the population was passive, suggesting that a group of neurons, even though they participate in the primary burst, can escape subsequent recruitment by nonsynaptic mechanisms. Passive neurons have not previously been reported in in vitro models of ictal activity. Among the three types of afterdischarges observed, type I was the most common. Here, afterdischarge coincided with a voltage-dependent plateau, which was sensitive to Na⁺ channel blockade. Intracellular QX314 abolished the plateau without blocking the fast APs. Together, these findings suggest that $I_{\text{NaP}}$, intrinsic to the pyramidal neurons (French et al. 1990), is the main charge carrier for the plateau. Plateaus could not be evoked by current injection in the absence of population activity, and were unaltered by temporal separation from the primary burst (Fig. 5), which indicates that their expression depends on factors specifically related to ictal-like population activity. Such factors could likely be ion concentration changes affecting Na⁺ channels (Somjen and Müller 2000; Yue et al. 2005) or enhanced transfer of signals through gap junctions. Isolated plateaus were generally seen to be initiated with maximal amplitude after a single AP, suggesting that they were independent of high-frequency firing causing summation of ADPs. This observation does not, however, exclude that ADP summation in a subset of neurons could be an important early step in initiating the ictal-like population activity. The marked inhibition of the ictal-like phase by TTX and phenytoin, at the concentrations used here, is consistent with an important role of $I_{\text{NaP}}$ in Cs⁺-induced
ictogenesis. In this respect the Cs\textsuperscript+ model is comparable to a number of other in vitro epilepsy models (Bikson et al. 2002; Kager et al. 2007; Mantegazza et al. 2010; Segal and Douglas 1997; Stafstrom 2007; Yaari and Beck 2002). Although the present study does not exclude the involvement of voltage-gated Ca\textsuperscript{2+} currents, previous evidence suggests that L-type Ca\textsuperscript{2+} currents make no contribution to the Cs-FP (Skov et al. 2005). The possibility of influence from other Ca\textsuperscript{2+} channels is difficult to evaluate directly, as the Cs-FP is abolished during general blockade of Ca\textsuperscript{2+} channels. However, observations on Cs\textsuperscript{+}-induced spontaneous field bursts, which are independent of synaptic transmission and which bear some resemblance to the ictal-like phase of the Cs-FP, show little effect of Cd\textsuperscript{2+} (200 \mu M)/Ni\textsuperscript{2+} (300 \mu M) on the amplitude but an altered frequency and duration of these events (Kraglund et al. 2010). While this finding might indicate some role for Ca\textsuperscript{2+} currents in modifying epileptiform activity, a detailed account of such influence, in particular its relationship to the $I_{\text{NaP}}$, is still missing.

The results with Na\textsuperscript{+} channel blockers indicate that the subset of neurons displaying type I behavior are pivotal for generating the ictal-like phase. In support of this, we observed in these neurons that APs, generated during the high-frequency bursts, were preceded by depolarizing rather than hyperpolarizing prepotentials, and a significant proportion of these occurred out of synchrony with the population spikes. Both findings indicate that the firing is subjected to an “intrinsic” drive. Most APs did, however, occur in timing with the negative slope region of the population spikes, suggesting that they contribute to the leading action current shaping the population spikes (Varona et al. 2000). These observations all point to neurons expressing type I behavior as playing a leading role in the synchronization process.

In the absence of a voltage-dependent plateau, the discharge fell into two categories. Type II discharge was slow and unrelated to population spikes, suggesting that these neurons were not directly engaged by the population activity. Type III discharge was strictly confined to a period of a few milliseconds around the peak of individual population spikes and

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**Fig. 9.** Blockade of the M current accelerates the development of epileptiform activity. A: firing in response to threshold stimulation before (control) and after 30-min perfusion with linopirdine (Lin, 10 \mu M). B: extracellular (top) and intracellular (bottom) recordings of evoked response taken after 30-min exposure to 10 \mu M linopirdine and after subsequent perfusion of Cs\textsuperscript+ for 12 and 20 min (Lin/Cs\textsuperscript{+}). Note the typical type I behavior of the penetrated pyramidal neuron. C: histogram of the average area of the negative phase of the Cs-FP after 30-min exposure to Cs\textsuperscript+ with (Cs\textsuperscript+/Lin, $n = 5$) or without (Cs\textsuperscript{+}, $n = 13$) preincubation in linopirdine. RMP: $-72 \text{ mV}$ (A), $-68 \text{ mV}$ (B).
showed a robust presence of a preceding hyperpolarizing prepotential. The hyperpolarizing prepotential is indicative of a passive neuron being recruited by field interactions (Haas and Jefferys 1984; Richardson et al. 1984; Taylor and Dudek 1982, 1984; Traub et al. 1985). Note that the prepotential results from currents derived from the extracellular electrical field of the population spike and is smaller than the population spike. It therefore represents a depolarization of the transmembrane potential in the soma, as would be revealed by subtraction of the intra- and extracellular voltage records (not shown). These findings suggest that type III behavior essentially depends on recruitment and that afterdischarge in this subgroup of neurons is directly shaped by the population activity.

Detailed accounts of the firing behavior of individual neurons during ictal events are sparse in the literature, but available results from in vitro studies suggest a homogeneous neuronal response reminiscent of the type I behavior described here (Anderson et al. 1986; Avoli et al. 1993; Bikson et al. 2002, 2003; Chesnut and Swann 1988; Köhling et al. 2000; Konnerth et al. 1986; Rafiq et al. 1993, 1995). In one study of high-[K+]o-induced ictal events, two types of discharge have been reported, which may seem equivalent to type I and type III behavior (Jensen et al. 1994; Jensen and Yaari 1997). Comparison with these earlier studies is, however, difficult as the demonstration of a voltage-dependent plateau underlying afterdischarge has not previously been used as a classifying criterion. To our knowledge, the present study is therefore the first report of a heterogeneous population behavior in an in vitro slice preparation. Interestingly, heterogeneous behavior is reported in a number of in vivo studies. During penicillin-induced seizures in cat cerebral cortex, 40% of neurons were found to be “active” (displaying prolonged burst firing), 37% were classified as “passive” (became active only in the late phases of the seizure), 10% could not be classified, and 4% were inhibited (Matsumoto and Marsan 1964). In another study of spontaneous seizures in cat cerebral cortex, two populations of neurons were described: regular firing neurons (77%) and fast-rhythmic-bursting neurons (23%) (Steriade et al. 1998). Wyler et al. (Wyler et al. 1975, 1978; Wyler and Ward 1986) found three groups of neurons in chronic epileptic foci in monkey cerebral cortex: group I (10%) were highly epileptic with burst firing, group II (40%) were weakly epileptic with a mixture of variable burst firing and regular firing, and group III (50%) were regular firing. Evidence from human temporal lobe seizures indicates that a relatively small proportion of neurons change their firing pattern during seizures (Babb et al. 1987). The authors estimate that in subclinical seizures only ~7% of neurons increase their firing, 14% show increased firing during clinical seizures with auras and changed consciousness, and 37% participate in clinical seizures with lost consciousness, movements, and postictal confusion. Collectively, these results indicate that a high diversity of neuronal response patterns is a
common feature of in vivo ictal events. In this respect, the in vitro ictal-like events induced in the presence of Cs⁺ seem to emulate the conditions found during in vivo ictogenesis.

**Possible reasons for differential neuronal behavior.** We found no evidence of a correlation between the shape or intensity of the epileptiform field activity and the response of the individual neurons. In particular, we observed marked epileptiform field activity in the vicinity of the intracellular electrode even when neurons were “passive” (Fig. 4). Type II behavior often coincided with little or no synchronized field activity, but this was not always the case (Fig. 3). Hence, the data here give no indication that the type of discharge depends on the position of the neuron with respect to areas with high versus low epileptic activity. Furthermore, as the basic membrane properties of neurons adopting different afterdischarge behaviors were similar (Table 1), it seems unlikely that the various afterdischarge behaviors reflect variations in the quality of the recordings. When impaled in the presence of Cs⁺, those neurons that adopted type II and III behavior were more depolarized compared with neurons adopting type I behavior and “passive” neurons (Table 2). However, it is very unlikely that RMP is a key determinant of the firing behavior since manipulation of membrane potential did not alter the phenotype. Our results show a change in membrane potential and $R_m$ that parallels the development of the Cs-FP (Fig. 1). Such changes, albeit modest, will contribute to the ictogenesis by raising the overall excitability of the pyramidal neurons. However, when comparing the changes observed in neurons adopting type I and non-type I behavior, there was no obvious difference.

It is well established that CA1 pyramidal neurons can be divided into “bursters” and regular firing neurons based on their intrinsic firing properties (Jensen et al. 1994; Schwartzkroin 1975; Yue et al. 2005). In both in vivo and in vitro models, the ratio of “bursters” generally increases in conjunction with the development of epileptiform activity (Chagnac-Amitai and Connors 1989; Church and McLennan 1989; Jensen et al. 1994; Sanabria et al. 2001; Szente and Boda 1994; Yamada and Bilkey 1991). It is therefore conceivable that neurons with intrinsic bursting capability would be more likely to adopt type I behavior. However, our results do not support this (Fig. 8). Furthermore, the induction of ictal-like activity in the presence of Cs⁺ was not associated with an increase in the number of “bursters.” Therefore, the intrinsic firing properties do not appear to be decisive for the type of afterdischarge adopted.

The size of the ADP is determined by the ratio between $I_{NaP}$ and $I_M$ (Yue and Yaari 2004), and any imbalance in favor of $I_{NaP}$ facilitates the induction of intrinsic bursting behavior (Azouz et al. 1996; Yue and Yaari 2004; Yue et al. 2005). Once initiated, bursting is prevented from becoming “run-away” activity by excess activation of the slow Ca²⁺-activated K⁺ current (Yue and Yaari 2006). As type I behavior strongly depends on $I_{NaP}$, it is possible that it is promoted by an imbalance between $I_{NaP}$ and $I_M$ in conjunction with a Cs⁺-induced blockade of the slow Ca²⁺-activated K⁺ current, as described previously (Skov et al. 2009). The observed effects of linopirdine show that type I behavior is antagonized by $I_M$, and enhancement of $I_M$ with retigabine blocked ictal-like activity altogether. This suggests a very strong regulatory impact of $I_M$ and raises the possibility that inhibition of $I_M$ is necessary for the expression of type I behavior. At the concentration used here, linopirdine also suppresses the fast Ca²⁺-activated K⁺ current ($I_C$) (Schnee and Brown 1998; Yue and Yaari 2004). However, selective inhibition of $I_C$ does not enhance the ADP nor does it convert regular firing neurons into bursters (Yue and Yaari 2004), suggesting that this current has little influence on the expression of $I_{NaP}$. It seems unlikely, therefore, that a reduction in $I_C$ could contribute significantly to the linopirdine-induced promotion of type I behavior.

Extracellular Cs⁺ has been reported to reduce $I_M$ in a voltage-dependent manner (Block and Jones 1996; Coggan et al. 1994). The Cs⁺-induced ADP amplification observed to correlate with type I behavior (Fig. 8) occurred without an increase in intrinsic bursting, which is unexpected if the mechanism was a simple blockade of $I_M$ (Jensen et al. 1996; Yue et al. 2005). The limited effect of Cs⁺ on the ADP in non-type I neurons is also not accounted for. This conflicting evidence points to a complex influence from several factors (one of which may be $I_M$ inhibition) to determine the firing behavior. The apparent all-or-none presence of the $I_{NaP}$-dependent plateau (as opposed to the ubiquitous ADP) suggests that a “threshold” needs to be crossed in order for a neuron to express type I behavior. This seems equivalent to the state transition from simple to self-regenerative afterdischarge shown in model studies to depend on small variations in $I_{NaP}$ conductance level (Kager et al. 2007). The altered excitability and ion distribution imposed by the primary burst may have variable impact in different neurons and hence determine whether the balance is tipped in favor of plateau generation.

**Pacemakers versus followers.** On the basis of their studies of in vivo seizure activity Wyler et al. (Wyler et al. 1975, 1978; Wyler and Ward 1986) proposed that neurons within an epileptic focus display a spectrum of behavior ranging from normal to highly epileptic, and the latter would act as pacemakers for the focus. These pacemaker neurons maintain focal epileptogenesis and produce ictal events by recruiting surrounding neurons, and once a “critical mass” is reached, ictogenesis becomes regenerative and propagating. The authors furthermore suggest that the onset of seizures depends on several factors such as synaptic activation, alterations of neuronal properties, and abnormalities of the environment, e.g., reactive gliosis. This model for ictogenesis finds support in studies of firing patterns during clinical temporal lobe seizures (Babb et al. 1987). Collectively, our present and previous results indicate that this model could also represent a blueprint for Cs⁺-induced ictogenesis (Andreassen et al. 2007; Skov et al. 2005, 2009). In our experiments, type I and type III neurons display the hallmarks of being pacemakers and followers, respectively (see discussion above), and these two groups together would therefore seem to provide the “critical mass” necessary for ictogenesis to become regenerative. The size of the “critical mass” (which by definition is a threshold value) cannot be determined from the present data, but the aforementioned two types of neurons constitute approximately two-thirds (44% and 18%) of the population, which would then be the ceiling level. The residual one-third of the population is composed of “passive” and type II neurons. The low-frequency random firing of type II neurons will contribute to increasing $K^+_M$ and thus to the hyperexcitability of the circuitry. The observation that increasing the number of neurons with type I behavior, by blocking $I_M$, did not significantly change the
already established Cs-FP indicates that, once the “critical mass” has been reached, the amount of activity is near-maximal. Conversely, enhancing \( I_M \) (and thus presumably lowering the number of type I neurons) could bring the “active” population below the “critical mass” and thereby prevent the process from entering a regenerative stage.

**Summary and conclusions.** We have described here in an in vitro model of ictal-like afterdischarge a highly heterogeneous response pattern among individual neurons. The characteristics of these patterns seem to suggest that neurons, instead of being distributed along a continuous scale of “excitability,” are divided into distinct groups (types), of which we have here identified four. Among these phenomenological types it seems that a functional designation can be inferred for some of them, such as pacemakers (type I) and followers (type III). It is noteworthy that this division into behavioral types only holds for the ictal-like phase, as we observed a uniform response during the preceding interictal-like phase. This suggests that it is events occurring in the transition between the two phases that determines the distribution of neurons into different types. The conditioning effect of the interictal-like activity is likely coupled to changes in the extracellular environment rather than to events set in motion in individual neurons. It will take further studies to identify which factors (likely multiple) are important for determining the different types of discharge. One element of significance for type I activity seems to be the balance between \( I_{NaP} \) and \( I_K \), which is in accordance with previous reports (Azouz et al. 1996; Yue and Yaari 2004; Yue et al. 2005). A particular problem appears to be the lack of correlation between intrinsic bursting and pacemaker behavior (type I), which is clearly at variance with previous suggestions (Chagnac-Amitai and Connors 1989; Connors 1984; Gutnick et al. 1982; Jensen and Yaari 1997; McCormick and Contreras 2001; Prince 1985; Sanabria et al. 2001; Steriade et al. 1998; Szente and Boda 1994; Traub et al. 1999; Traub and Wong 1982; Yaari and Beck 2002). These were based on an observed increase in the number of “bursters,” which we failed to detect in the present study. The latter discrepancy is likely related to differences in the models used, indicating that more insight into the impact of different regulatory influences on ictogenesis in general could be obtained in future in vitro studies by comparing neuronal behavior across different epilepsy models.

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No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**

Author contributions: M.A. and S.N. conception and design of research; M.A. performed experiments; M.A. and S.N. analyzed data; M.A. and S.N. interpreted results of experiments; M.A. prepared figures; M.A. and S.N. drafted manuscript; M.A. and S.N. edited and revised manuscript; M.A. and S.N. approved final version of manuscript.

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