Chloride-Cotransport Blockade Desynchronizes Neuronal Discharge in the “Epileptic” Hippocampal Slice

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Hochman, Daryl W. and Philip A. Schwartzkroin. Chloride-cotransport blockade desynchronizes neuronal discharge in the “epileptic” hippocampal slice. J. Neurophysiol. 83: 406-417, 2000. Antagonism of the chloride-cotransport system in hippocampal slices has been shown to block spontaneous epileptiform (i.e., hypersynchronized) discharges without diminishing excitatory synaptic transmission. Here we test the hypotheses that chloride-cotransport blockade, with furosemide or low-chloride (low-[Cl\(^-\)]) medium, desynchronizes the firing activity of neuronal populations and that this desynchronization is mediated through nonsynaptic mechanisms. Spontaneous epileptiform discharges were recorded from the CA1 and CA3 cell body layers of hippocampal slices. Treatment with low-[Cl\(^-\)] medium led to cessation of spontaneous synchronized bursting in CA1 $\geq$5-10 min before its disappearance from CA3. During the time that CA3 continued to burst spontaneously but CA1 was silent, electrical stimulation of the Schaffer collaterals showed that hyperexcited CA1 synaptic responses were maintained. Pairs of intracellular recordings from CA1 pyramidal cells showed that during low-[Cl\(^-\)] treatment, the timing of action potential discharge became desynchronized; desynchronization was identified with phase lags in firing times of action potentials between pairs of neurons as well as a broadening and diminution of the CA1 field amplitude. Continued exposure to low-[Cl\(^-\)] medium increased the degree of the firing-time phase shifts between CA1 and CA3 pyramidal cells until the epileptiform CA1 field potential was abolished completely. Intracellular recordings during 4-aminopyridine (4-AP) treatment showed that prolonged low-[Cl\(^-\)] exposure did not diminish the frequency or amplitude of spontaneous postsynaptic potentials. CA3 antidromic responses to Schaffer collateral stimulation were not significantly affected by prolonged low-[Cl\(^-\)] exposure. In contrast to CA1, paired intracellular recordings from CA3 pyramidal cells showed that chloride-cotransport blockade did not cause a significant desynchronization of action potential firing times in the CA3 subregion at the time that CA1 synchronous discharge was blocked but did reduce the number of action potentials associated with CA3 burst discharges. These data support our hypothesis that the anti-epileptic effects of chloride-cotransport antagonism in CA1 are mediated through the desynchronization of population activity. We hypothesize that interference with Na\(^+\),K\(^+\),2Cl\(^-\) cotransport results in an increase in extracellular potassium ([K\(^+\)]) that reduces the number of action potentials that are able to invade axonal arborizations and varicosities in all hippocampal subregions. This reduced efficacy of presynaptic action potential propagation ultimately leads to a reduction of synaptic drive and a desynchronization of the firing of CA1 pyramidal cells.

INTRODUCTION

Synchronization of spontaneous neuronal firing activity is thought to be an important feature of a number of normal and pathological processes in the CNS. Examples include synchronized oscillations of population activity such as gamma rhythms in neocortex, which are thought to be involved in cognition (Singer and Gray 1995), and theta rhythm in hippocampus, which is thought to play roles in spatial memory and in the induction of synaptic plasticity (Huerta and Lisman 1995, 1996; O’Keefe 1993). Epileptiform activity is identified with spontaneously occurring hypersynchronized discharges of neuronal populations (Traub and Jefferys 1994). Most research on the processes underlying the generation and maintenance of spontaneous synchronized activity has focused on synaptic mechanisms (Abeles et al. 1994; Bland and Colom 1993; McNamara 1994; Stanford et al. 1998; Traub and Miles 1991; Williams and Kauer 1997). However, much data have supported the notion that nonsynaptic mechanisms also could play important roles in the modulation of synchronization in normal and pathological activities in the CNS (Andrew 1991; Dudek and Traub 1989; Faber and Korn 1989; Jeffreys 1995).

In previous reports, we have shown that inhibition of cation chloride cotransport, either by a reduction of extracellular chloride ([Cl\(^-\)]\(_{o}\)) or by treatment with transporter antagonists such as loop-diuretics (furosemide and bumetanide), blocks spontaneous epileptiform activity (Hochman et al. 1995, 1999; Schwartzkroin et al. 1998). Further, because furosemide and low-[Cl\(^-\)]\(_{o}\) effectively blocked spontaneous bursting elicited by a variety of treatments that work through a spectrum of different synaptic mechanisms, it appears that chloride-cotransport antagonism affects general processes critical for maintenance of spontaneous synchronized neuronal activity. Several observations from our previous studies on hippocampal slices suggest that the anti-epileptic effects of furosemide and low-[Cl\(^-\)]\(_{o}\) are mediated by nonsynaptic mechanisms: excitatory synaptic transmission (as measured by field responses of area CA1 to Schaffer collateral stimulation) is not diminished; spontaneous field shifts induced by low-Ca\(^{2+}\) treatment—a type of epileptiform activity thought to be mediated through nonsynaptic mechanisms (Jefferys and Haas 1982; Taylor and Dudek 1982)—can be blocked; and activity-evoked intrinsic optical changes of the tissue are blocked concomitant with the blockade of spontaneous epileptiform activity, suggesting that changes in the volume fraction of the extracellular space (ECS) or ionic fluxes associated with cell volume regulation might be related to the anti-epileptic effects of furosemide (Holthoff and Witte 1996; MacVicar and Hochman 1991).

Our previous study (Hochman et al. 1999) suggested that it was the blockade of the Na\(^+\),K\(^+\),2Cl\(^-\) cotransporter that was critical to the anti-epileptic effects of chloride-cotransport antagonism. Further, prolonged exposure of the tissue to lower-[Cl\(^-\)] medium led to the cessation of spontaneous epileptiform discharges without compromising excitatory synaptic transmission. Here we test the hypotheses that chloride-cotransport blockade, with furosemide or low-chloride (low-[Cl\(^-\)]) medium, desynchronizes the firing activity of neuronal populations and that this desynchronization is mediated through nonsynaptic mechanisms. Spontaneous epileptiform discharges were recorded from the CA1 and CA3 cell body layers of hippocampal slices. Treatment with low-[Cl\(^-\)] medium led to cessation of spontaneous synchronized bursting in CA1 $\geq$5-10 min before its disappearance from CA3. During the time that CA3 continued to burst spontaneously but CA1 was silent, electrical stimulation of the Schaffer collaterals showed that hyperexcited CA1 synaptic responses were maintained. Pairs of intracellular recordings from CA1 pyramidal cells showed that during low-[Cl\(^-\)] treatment, the timing of action potential discharge became desynchronized; desynchronization was identified with phase lags in firing times of action potentials between pairs of neurons as well as a broadening and diminution of the CA1 field amplitude. Continued exposure to low-[Cl\(^-\)] medium increased the degree of the firing-time phase shifts between CA1 and CA3 pyramidal cells until the epileptiform CA1 field potential was abolished completely. Intracellular recordings during 4-aminopyridine (4-AP) treatment showed that prolonged low-[Cl\(^-\)] exposure did not diminish the frequency or amplitude of spontaneous postsynaptic potentials. CA3 antidromic responses to Schaffer collateral stimulation were not significantly affected by prolonged low-[Cl\(^-\)] exposure. In contrast to CA1, paired intracellular recordings from CA3 pyramidal cells showed that chloride-cotransport blockade did not cause a significant desynchronization of action potential firing times in the CA3 subregion at the time that CA1 synchronous discharge was blocked but did reduce the number of action potentials associated with CA3 burst discharges. These data support our hypothesis that the anti-epileptic effects of chloride-cotransport antagonism in CA1 are mediated through the desynchronization of population activity. We hypothesize that interference with Na\(^+\),K\(^+\),2Cl\(^-\) cotransport results in an increase in extracellular potassium ([K\(^+\)]) that reduces the number of action potentials that are able to invade axonal arborizations and varicosities in all hippocampal subregions. This reduced efficacy of presynaptic action potential propagation ultimately leads to a reduction of synaptic drive and a desynchronization of the firing of CA1 pyramidal cells.

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spontaneous synaptic activity in the CA1 and CA3 regions of neuronal and stimulation-evoked action potential discharges and spontaneous synaptic activity in the CA1 and CA3 regions of hippocampal slices.

**METHODS**

Slices were prepared from Sprague-Dawley adult rats as in previous studies (Hochman et al. 1995, 1999). Transverse hippocampal slices 400-µm thick were cut with a vibrating cutter. Slices typically contained the entire hippocampus and subiculum. After cutting, slices were stored in an oxygenated holding chamber at room temperature for ≥1 h before recording. All recordings were acquired in an interface chamber with oxygenated (95% O₂-5% CO₂) artificial cerebrospinal fluid (ACSF) at 34–35°C. Normal ACSF contained (in mmol/l) 124 NaCl, 3 KCl, 1.25 NaH₂PO₄, 1.2 MgSO₄, 26 NaHCO₃, 2 CaCl₂, and 10 dextrose.

Sharp-electrodes for intracellular recordings from CA1 and CA3 pyramidal cells were filled with 4 M potassium acetate. Field recordings from the CA1 and CA3 cell body layers were acquired with low-resistance glass electrodes filled with 2 M NaCl. For stimulation of the Schaffer collateral or hilar pathways, a small monopolar tungsten electrode was placed on the surface of the slice. Spontaneous and stimulation-evoked activities from field and intracellular recordings were digitized (Neurocorder, Neurodata Instruments, New York, NY) and stored on videotape. AxoScope software (Axon Instruments) on a personal computer was used for off-line analysis of data.

In some experiments, normal or low-[Cl⁻]₀ medium was used containing bicuculline (20 µM), 4-amino pyridine (4-AP; 100 µM), or high-K⁺ (7.5 or 12 mM). In all of our experiments, low-[Cl⁻]₀, 7.5 or 12 mM NaCl with Na⁺-glucuronate (Sigma) (see Hochman et al. 1999 for rationale). All solutions were prepared so that they had a pH of ~7.4 and an osmolarity of 290–300 mosmol at 35°C at equilibrium from carboxygenation with 95% O₂-5% CO₂.

After placement in the interface chamber, slices were superfused at ~1 ml/min. At this flow-rate, it took 8–10 min for changes in the perfusion media to be completed. All of the times reported here have taken this delay into account and have an error of approximately ±2 min. This accuracy was sufficient for the purposes of this study.

**RESULTS**

**Timing of cessation of spontaneous epileptiform bursting in areas CA1 and CA3**

The relative contributions of the factors that modulate synchronized activity vary between areas CA1 and CA3. These factors include differences in the local circuitry (Schwartzkroin 1993) and in region-specific differences in cell packing and volume fractions of the extracellular spaces (McBain et al. 1990). If the anti-epileptic effects of chloride-cotransport antagonism are due to a desynchronization in the timing of neuronal discharge, chloride-cotransport blockade might be expected to differentially affect areas CA1 and CA3. To test this hypothesis, we performed a set of experiments to characterize differences in the timing of the blockade of spontaneous epileptiform activity in areas CA1 and CA3 (Fig. 1).

Field activity was recorded simultaneously in areas CA1 and CA3. After about 30–40 min exposure to furosemide or low-chloride medium, spontaneous bursting ceased in area CA1 before the bursting ceased in area CA3 (cf. Fig. 1, A and B). The temporal sequence of events typically observed included an initial increase in burst frequency and amplitude of the spontaneous field events, then a reduction in the amplitude of the burst discharges that was more rapid in CA1 than in CA3. After CA1 became silent, CA3 continued to discharge for 5–10 min, until it too no longer exhibited spontaneous epileptiform events (Fig. 1C).

This temporal pattern of burst cessation was observed with all epileptiform-inducing treatments tested, regardless of whether the agent used for blockade of spontaneous bursting was furosemide or low-[Cl⁻]₀ medium. Throughout all stages of these experiments, stimulation of the Schaffer collaterals evoked hyperexcited field responses in both the CA1 and CA3 cell body layers. Immediately after spontaneous bursting was blocked in both areas CA1 and CA3, hypereexcited population spikes still could be evoked (Fig. 1C) as was reported previously (Hochman et al. 1999).

We considered the possibility that the observed cessation of bursting in CA1 before CA3 was an artifact of the organization of synaptic contacts between these areas relative to our choice of recording sites. If the cessation of bursting occurs in the different subregions of CA3 at different times, the results of the aforementioned set of experiments might arise not as a difference between CA1 and CA3 but rather as a function of variability in bursting activity across CA3 subregions. Therefore immediately after the spontaneous bursting ceased in CA1, we surveyed the CA3 field with a recording electrode. Recordings from several different CA3 locations (from the most proximal to the more distal portions of CA3), showed that all subregions of area CA3 were spontaneously bursting during this time that CA1 was silent (not shown).

**Effects of reduced extracellular chloride on the synchronization of CA1 and CA3 field population discharges**

The observations from the previous set of experiments suggested a temporal relationship between the exposure-time to low-[Cl⁻]₀ or furosemide-containing medium and the characteristics of the spontaneous burst activity. Further, this relationship was different between areas CA1 and CA3. To better characterize these temporal relationships, we compared the occurrences of CA1 action potentials and the population spike events in the field responses of CA1 and CA3 subfields during spontaneous and stimulation-evoked burst discharges. Figure 2 shows CA1 intracellular activity in relationship to spontaneous...
burst discharges (fields) during low-\([\text{Cl}^-]\) blockade of bicuculline-induced (20 \(\mu\text{M}\)) activity.

Intracellular recordings were obtained from CA1 pyramidal cells, with the intracellular electrode placed close (<100 \(\mu\text{m}\)) to the CA1 field electrode. The slice was stimulated every 20 s with single stimuli delivered to the Schaffer collaterals. After continuous spontaneous bursting was established for \(\approx 20\) min, the bathing medium was switched to bicuculline-containing low-chloride (21 \(\text{mM}\)) medium. After \(\approx 20\) min, the burst frequency and amplitude was at its greatest.

Simultaneous field and intracellular recordings during this time (Fig. 2A) showed that the CA1 field and intracellular recordings were synchronized closely with the CA3 field discharges. During each spontaneous discharge, the CA3 field response preceded the CA1 discharge by several milliseconds (Fig. 2A, left). During stimulation-evoked events, action potential discharges of the CA1 pyramidal cell were closely synchronized to both CA3 and CA1 field discharges (Fig. 2A, right).

With continued exposure to low-\([\text{Cl}^-]\) medium, the latency between the spontaneous discharges of areas CA1 and CA3 increased, with a maximum latency of 30–40 ms occurring after 30- to 40-min exposure to the bicuculline-containing low-chloride medium (Fig. 2B, left). During this time, the amplitude of both the CA1 and CA3 spontaneous field discharges decreased. Stimulation-evoked discharges during this time (Fig. 2B, right) closely mimicked the spontaneously occurring discharges in morphology and relative latency. However, the initial stimulus-evoked depolarization of the neuron [presumably, the monosynaptic excitatory postsynaptic potential (EPSP)] began without any significant increase in latency (Fig. 2B, bottom right). The time interval during which these data were acquired corresponds to the time interval shown at the beginning of the traces in Fig. 1B immediately before the cessation of spontaneous bursting in CA1.

After 40- to 50-min perfusion with low-\([\text{Cl}^-]\) medium, the spontaneous bursts were nearly abolished in CA1 but were unaffected in CA3. Schaffer collateral stimulation during this time showed that monosynaptically triggered responses of CA1 pyramidal cells occurred without any significant increase in latency but that stimulation-evoked field responses were almost abolished (Fig. 2C). The time interval during which these data were acquired corresponds to the moments immediately before the cessation of spontaneous bursting in CA3, shown at the beginning of the traces in Fig. 1C.

After prolonged exposure to low-\([\text{Cl}^-]\) medium, large increases (>30 ms) developed in the latency between Schaffer collateral stimulation and the consequent CA3 field discharge (Fig. 3, left). Eventually, no field responses could be evoked by Schaffer collater stimulation in either areas CA1 or CA3 (Fig. 3, right). However, action potential discharge from CA1 pyra-
midal cells, in response to Schaffer collateral stimulation, could be evoked with little change in response latency. Indeed, for the entire duration of our experiments (>2 h), action potential discharges from CA1 pyramidal cells could be evoked at short latency by Schaffer collateral stimulation. Further, although stimulation-evoked hyperexcited discharges of CA3 eventually were blocked after prolonged exposure to low-\([\text{Cl}^-]\)_o medium, the antidromic response in CA3 appeared to be preserved (Fig. 3, top).

**Effects of chloride-cotransport antagonism on the synchronization of burst discharges in CA1 pyramidal cells**

These foregoing data suggest that the disappearance of the field responses may be due to a desynchronization of the occurrence of action potentials among neurons. That is, although synaptically driven excitation of CA1 pyramidal cells was preserved, action potential synchrony among the CA1 neuronal population was not sufficient to summate into a measurable DC field response. To test this hypothesis, paired intracellular recordings of CA1 pyramidal cells were acquired simultaneously with CA1 field responses. In these experiments, both of the intracellular electrodes and the field recording electrodes were placed within 200 μm of one another.

During the period of maximum spontaneous activity induced by bicuculline-containing low-\([\text{Cl}^-]\)_o medium, recordings showed that action potentials between pairs of CA1 neurons and the CA1 field discharges were synchronized tightly both during spontaneous and stimulation-evoked discharges (Fig. 4A). After continued exposure to low-chloride medium, when the amplitude of the CA1 field discharge began to broaden and diminish in amplitude, both spontaneous and stimulation-evoked discharges showed a desynchronization in the timing of the occurrences of action potentials between pairs of CA1 neurons, and between the action potentials and the field responses (Fig. 4B). This desynchronization was coincident with the suppression of CA1 field amplitude. By the time that spontaneous bursting in CA1 ceased, a significant increase in latency had developed between Schaffer collateral stimulation and CA1 field discharge (Fig. 4C). At this time, paired intracellular recordings showed a dramatic desynchronization in the timing of action potential discharges between pairs of neurons and between the occurrence of action potentials and the field discharges evoked by Schaffer collateral stimulation.

It is possible that the observed desynchronization of CA1 action potential discharge is due to the randomization of mechanisms necessary for synaptically driven action potential generation, such as a disruption in the timing of synaptic release or random conduction failures at neuronal processes. If this was
the case, then one would expect that the occurrence of action potentials between a given pair of neurons would vary randomly with respect to one another from stimulation to stimulation. We tested this hypothesis by comparing the patterns of action potential discharge of pairs of neurons between multiple consecutive stimuli of the Schaffer collaterals (data not shown). During each stimulation event, the action potentials occurred at nearly identical times with respect to one another and showed an almost identical burst morphology from stimulation to stimulation. We also checked to see whether the occurrence of action potentials between a given pair of neurons, during spontaneous field discharges, was fixed in time. The patterns of action potential discharges from a given pair of CA1 neurons was compared between consecutive spontaneous field bursts during the time when the occurrence of action potentials was clearly desynchronized (e.g., Fig. 4). Just as in the case of stimulation-evoked action potential discharge described earlier, the action potentials generated during a spontaneous population discharge occurred at nearly identical times with respect to one another and showed a nearly identical burst morphology from one spontaneous discharge to the next.

**Synchronization of burst discharges in CA3 pyramidal cells**

The results of the previous experiments support the hypothesis that spontaneous bursting ceases in CA1 because of a low-[Cl\textsuperscript{–}]\textsubscript{o}–induced desynchronization of the action potentials of the CA1 pyramidal cell population. To test whether a desynchronization of action potential discharge also underlies the cessation of bursting in CA3, we carried out a series of similar experiments with paired intracellular and field recordings acquired in area CA3 and a stimulating electrode placed on the hilus for activation of the mossy fiber afferent input pathway (Fig. 5). In addition to testing our hypothesis regarding the desynchronization of neuronal activity, we were interested in whether chloride-cotransport antagonism affected a different hippocampal circuit (in this case, the hilus-to-CA3 circuit) in a manner similar to its effects on the CA3-to-CA1 circuit.

Slices (n = 6) were perfused with high-K\textsuperscript{+} medium (8 or 12 mM) until continuous spontaneous bursting had been elicited in CA3 for ≥20 min and thereafter bathed with high-[K\textsuperscript{+}]\textsubscript{o} medium containing furosemide (2.5 mM). As previously reported (Hochman et al. 1999), an initial period of increased burst amplitude was observed in CA3 after exposure to furosemide. Simultaneous field and paired intracellular recordings during this period of increased excitability showed a tight synchronization in the occurrence of action potentials between a given pair of neurons, and between the occurrence of action potentials and the morphology of the CA3 field burst (Fig. 5A). Both spontaneously occurring discharges (Fig. 5A, left) and stimulation-evoked discharges (Fig. 5A, right) showed similar synchronization. After 40 min of furosemide exposure, immediately before the complete blockade of spontaneous bursting, no changes in the synchronization of neuronal activity was observed during either spontaneously occurring (Fig. 5B, left) or stimulation-evoked (Fig. 5B, right) discharges. Note the nearly identical morphology of the pattern of action potential firing patterns between the spontaneous and stimulation-
evoked discharges. Most importantly, note that the number of action potentials (1–3) associated with each discharge after prolonged furosemide exposure is significantly less than the number of action potentials (6–10) associated with discharges during the peak of spontaneous burst activity. Although we did not have an electrode in the CA1 region during these experiments, it is reasonable to assume (based on previous experiments) that the CA3 responses illustrated in Fig. 5 were acquired during a time when spontaneous bursting in CA1 had ceased.

As in the case of the previous experiments that focused on the CA1 subregion, we were interested in whether the suppression of activity in CA3 was due to a randomization of processes involved in the generation of action potentials. In all our experiments, we noted for comparison the patterns of action potential discharge between a given pair of neurons during a consecutive series of discharges (data not shown). Consecutive spontaneous discharges, immediately before (<2 min) the complete furosemide-blockade of spontaneous bursting,

**FIG. 5.** Effects of furosemide on the synchronization of CA3 discharges. Spontaneous bursting was elicited in hippocampal slices by exposure to high-[K+]o medium (12 mM; n = 6). Simultaneously, intracellular recordings from pairs of CA3 pyramidal cells and a field recording from the CA3 cell body layer were acquired. A: during the peak of spontaneous burst activity (initial exposure to furosemide-containing high-[K+]o medium), action potentials were synchronized between pairs of neurons and with the negative peaks of the field response. B: after prolonged exposure to furosemide (40 min), the occurrence of action potentials appeared to be desynchronized with respect to one another and with respect to the negative peaks of the field response.
alane-2,3-dione (CNQX), 50 μM]. The field electrode was adjusted to optimize the amplitude of the antidromic response in the CA3b region.

Figure 6A shows the orthodromic CA1 response (top) and the antidromic CA3 response (bottom) to Schaffer stimulation in normal medium. After blockade of synaptic transmission (bicuculline, 20 μM; 2-amino-5-phosphonovaleric acid (APV), 100 μM; 6-cyano-7-nitroquininalene-2,3-dione (CNQX), 50 μM), the slice was exposed to low-[Cl\textsuperscript{-}]\textsubscript{o} (7 mM) medium. Even after prolonged exposure (>90 min), no significant increase in latency developed between stimulation and the antidromic field response.

Effects of low-chloride treatment on spontaneous synaptic activity

It is possible that the anti-epileptic effects associated with chloride-cotransport antagonism are mediated by some action on transmitter release. Blockade of chloride cotransport could alter the amount or timing of transmitter released from terminals, thus affecting neuronal synchronization. To test whether low-[Cl\textsuperscript{-}]\textsubscript{o} exposure affected mechanisms associated with transmitter release, intracellular CA1 responses were recorded simultaneously with CA1 and CA3 field responses during a treatment that dramatically increases spontaneous synaptic release of transmitter from presynaptic terminals (Fig. 7). Increased spontaneous release of transmitter was induced by treatment with 4-AP (100 μM). After 40-min exposure to 4-AP-containing medium, spontaneous synchronized burst discharges were recorded in areas CA1 and CA3. Switching to 4-AP-containing low-[Cl\textsuperscript{-}]\textsubscript{o} medium led initially, as was shown previously, to enhanced spontaneous bursting (20 min...
after exposure to low-[Cl\(^-\)]\(_o\) medium; Fig. 7A). High-gain intracellular recordings showed that high-amplitude spontaneous synaptic activity was elicited by 4-AP treatment (Fig. 7A, bottom). Further exposure to low-chloride medium blocked spontaneous burst discharge in CA1, although CA3 continued to discharge spontaneously (Fig. 7B, top). At this time, CA1 intracellular recordings showed that spontaneous synaptic noise was increased further and remained so for prolonged exposure times to 4-AP-containing low-chloride medium (Fig. 7C). These data suggest that mechanisms responsible for synaptic release from terminals are not adversely affected by low-chloride exposure in a manner that could explain the blockade of 4-AP-induced spontaneous bursting in CA1. These results also eliminate the possibility that the effects of low-[Cl\(^-\)]\(_o\) exposure are due to alterations in CA1 dendritic properties that would compromise their efficiency in conducting PSPs to the soma.

Finally, in six experiments, we observed the affects of furosemide treatment on synaptic activity measured with paired intracellular recordings of CA3 pyramidal cells in slices in which spontaneous bursting activity was elicited by exposure to high-K\(^+\)-medium (8 or 12 mM; data not shown). In these experiments, hyperpolarizing current was injected into the cells to prevent action potential discharge between bursts so that the EPSPs could be distinguished more easily. During the period of spontaneous bursting, large EPSPs that were synchronized between pairs of neurons, and synchronized with small deflections in the field recording, were recorded routinely. The amplitude, frequency, and degree of EPSP synchrony were maximal just after a spontaneous burst and diminished just before the subsequent burst. After prolonged exposure to furosemide, which blocked all spontaneous bursting (>40 min), the frequency and amplitude of the EPSPs were significantly reduced, although infrequent synchronized EPSPs still could be distinguished.

**Discussion**

The goal of this study was to test the hypotheses that blockade of chloride-cotransport results in a desynchronization of neuronal activity and that the anti-epileptic effects of chloride-cotransport blockade are independent of direct affects on excitatory synaptic transmission. Presumably extracellular field recordings from the cell body layers of hippocampal slices represent some statistical average of the activity of the population of neurons near the recording electrode. We suppose that a necessary degree of temporal synchronization of action potential firing is required in a geometrically organized neuronal population so that dipoles summate into a detectable field response (Andersen 1975; Faber and Korn 1989). Given this interpretation, several observations from our previous studies (Hochman et al. 1995, 1999) motivated the first hypothesis—that chloride-cotransport blockade leads to a desynchronization of population neuronal activity. First, tissue exposure to furosemide or low-[Cl\(^-\)]\(_o\) medium blocked spontaneous epileptiform (i.e., synchronized) burst discharges in the cell body layers of areas CA1 and CA3. Second, during prolonged exposure to low-[Cl\(^-\)]\(_o\) medium, action potential discharge capabilities of CA1 pyramidal cells remained intact even though the field recording was silent. Our second hypothesis, that the anti-epileptic effects of chloride-cotransport blockade are independent of effects on transmitter release at the synapse, was motivated by the observations that furosemide and low-[Cl\(^-\)]\(_o\) blocked spontaneous field discharges but that hyperexcited CA1 field response still could be evoked by Schaffer collateral stimulation. These hyperexcited field responses to stimulation (indicated by multiple population spikes), seen during furosemide or low-[Cl\(^-\)]\(_o\) treatment, likely reflect diminished GABA\(_A\) inhibitory currents induced by the blockade of cation-chloride cotransport (Hochman et al. 1999; Misgeld et al. 1986; Thompson and Gähwiler 1989; Thompson et al. 1988).

**Loss of coherence in action potential firing times**

Several important aspects of the effects chloride-cotransport blockade on the firing times of CA1 neurons should be noted: 1) the loss in the precise timing of action potential firing between pairs of neurons was coincident with the blockade of spontaneous epileptiform activity, suggesting that this change in the timing of action potentials is involved in the anti-epileptic effects of chloride-cotransport blockade; 2) although a significant offset in the times of occurrences of action potentials between pairs of CA1 neurons developed with low-[Cl\(^-\)]\(_o\) exposure, the offset for a given pair of neurons remained constant with respect to one another during a fixed interval of the experiment. Thus rather than describing the consequence of low-[Cl\(^-\)]\(_o\) exposure as a loss of “synchronization,” it is more accurate to describe it as an induction of “fixed phase lags” in the timing of action potential firing between neurons in the CA1 population or as a transition from a state in which the firing patterns of the population of CA1 pyramidal cells are “coherent” to a state where they are “incoherent” (Block 1997). Before low-[Cl\(^-\)]\(_o\) exposure, the CA1 population of pyramidal cells fired action potentials in phase with one another and with the field, whereas after prolonged exposure, the firing of action potentials among the population became sufficiently incoherent so that the field response eventually was diminished below the threshold of detectability. We propose that the anti-epileptic effects of chloride-cotransport blockade in CA1 are a result of its action on the coherence of action potential firing.

**Changes in excitatory synaptic transmission**

Our observations are consistent with the hypothesis that a loss of coherence of CA1 action potential discharges, induced by low-[Cl\(^-\)]\(_o\) exposure, is independent of changes in excitatory transmission. First, even after the CA1 field response to Schaffer collateral stimulation had been blocked completely by prolonged exposure to low-[Cl\(^-\)]\(_o\) medium, Schaffer collateral stimulation still faithfully evoked monosynaptic EPSPs (and action potentials) with short latency. This observation suggests that the excitatory synaptic mechanisms associated with the stimulation of presynaptic fibers were still functional. Second, the magnitude and frequency of 4-AP-induced spontaneous transmitter release from presynaptic terminals did not appear to be decreased by low-[Cl\(^-\)]\(_o\) exposure. Indeed, the magnitude of the 4-AP-induced PSPs actually increased with prolonged exposure to low-[Cl\(^-\)]\(_o\) exposure. This result has two important implications for the interpretation of the effects of low-[Cl\(^-\)]\(_o\) exposure on the CA3-CA1 circuit: the mechanisms associated with the presynaptic release of transmitter are not affected in a way that could explain the blockade of spontaneous bursting in CA1, and transmission of PSPs from the dendritic regions to
the somata of CA1 cells are not affected. Finally, the characteristics of the low-[Cl\(^{-}\)]\(_o\)-induced phase lags in the timing of action potentials (described in the preceding text) are consistent with the interpretation that this loss of coherence in action potential timing is independent of affects on excitatory synaptic transmission. That is, if the observed phase shifting effects were due to changes associated with synaptic mechanisms [such as changes in the timing of transmitter release or changes in the kinetics of transmitter reuptake by synaptic vesicles (Diamond and Jahr 1997; Nicholls and Attwell 1990; Otma-khov et al. 1993; Rusakov and Kullmann 1998; Tong and Jahr 1994; Wolosker et al. 1996; Zador 1998)], it would be expected that the phase lags between pairs of neurons would vary randomly with respect to one another, from burst to burst; it would be unlikely that a given synapse would fail in exactly the same way from one stimulation trial to the next. However, the pattern of action potential discharges between pairs of neurons in our experiments remained nearly invariant between consecutive discharges, suggesting that a randomization of PSP mechanisms was not responsible for the generation of the observed phase lags.

During the time interval when CA1 field potentials were blocked by low-[Cl\(^{-}\)]\(_o\) exposure, while CA3 continued to generate spontaneous discharges, intracellular recordings from CA1 pyramidal cells showed that no PSPs were generated by the spontaneous presynaptic bursts even though electrical stimulation of the presynaptic fibers continued to evoke a discharge. We can interpret this result in a manner consistent with the preceding discussion: the disappearance of the spontaneously occurring CA1 field response, although consistent with the incoherence of action potential discharge, is due to a reduction in the ability of action potentials in CA3 neurons to invade the synaptic terminals. This conclusion is most clearly supported by the results of our 4-AP experiment (Fig. 7). During the time that spontaneous bursting ceased in CA1 (but continued in CA3), it was clear that the EPSPs evoked by the synchronized discharges of CA3 were not propagated to CA1 even though large-amplitude PSPs due to 4-AP-induced spontaneous transmitter release were clearly present. This observation suggests that there was a blockade of action potential propagation somewhere between the presynaptic CA3 cell and the synaptic terminal—but that the terminal itself was still capable of releasing transmitter. Further, this result suggests that this blockade affected only a fraction of the synapses because electrical stimulation of the presynaptic fibers was capable of evoking a maximal postsynaptic response. We propose that this presynaptic effect, related to a blockade of action potential invasion into synaptic terminals, is responsible for the changes in the timing of postsynaptic action potential generation.

In contrast to spontaneous activity, electrical stimulation of presynaptic axons was always sufficient to evoke synthetically driven discharges in CA1 (Fig. 3). Electrical stimulation of the Schaffer collaterals likely results in the simultaneous depolarization of all the axons within the vicinity of the stimulating electrode, thus initiating a highly synchronized discharge of action potentials in Schaffer collateral (and other) axons. In contrast, because low-[Cl\(^{-}\)]\(_o\) exposure caused a diminution of spontaneous CA3 discharges (discussed in the following text), spontaneous CA3 bursts undoubtedly represent a less powerful synaptic drive, involving a smaller number of axons. The ability of action potentials in presynaptic neurons to evoke a postsynaptic response during chloride-cotransport antagonism is likely dependent on a sufficiently large number of presynaptic neurons firing action potentials in phase with one another.

**Axonal conduction**

The results discussed so far suggest that the anti-epileptic effects of chloride-cotransport blockade in area CA1 are not due to postsynaptic actions because action potential generation and the propagation of PSPs from the dendrites to the soma in postsynaptic cells are not affected. Further, impairment of mechanisms associated with the release of synaptic transmitter does not seem to be involved. Because the ability of presynaptic CA3 pyramidal cells to fire action potentials is not impaired, it is likely that low-[Cl\(^{-}\)]\(_o\) exposure mediates its effects by affecting propagation of action potentials at some point between the soma and the terminals of CA3 pyramidal cells. To test whether this effect was mediated by decreasing the ability of CA3 axons to support action potentials, we examined the consequences of low-[Cl\(^{-}\)]\(_o\) exposure on the antidromic response of CA3 pyramidal cells (Fig. 6). Prolonged exposure to low-[Cl\(^{-}\)]\(_o\) medium did not significantly alter the antidromic response of CA3 to Schaffer collateral stimulation. This result rules out gross changes in action potential propagation by the major axonal branches of CA3 pyramidal cells. However, it may be that action potential propagation by smaller axonal arborizations, or the ability of action potentials to invade the axonal varicosities immediately before the synaptic terminals, is affected (Andersen 1975; Shepherd and Harris 1998; Westrum and Blackstad 1962).

We observed that during chloride-cotransport antagonism, CA3 continued to burst spontaneously for a brief period of time after bursting ceased in CA1. We did not observe any noticeable loss of coherence in CA3 action potential firing. Both of these observations imply that area CA3 is less vulnerable than CA1 to treatments that affect chloride cotransport. This difference between the responses of areas CA1 and CA3 may be due to the existence of local excitatory synaptic contacts between CA3 pyramidal cells; this could provide a more robust means for maintaining coherence in the firing of action potentials. It should be noted that after prolonged exposure to furosemide, CA3 neurons fired fewer action potentials during burst discharges. This finding suggests that the axons comprising the recurrent excitatory synaptic connections between CA3 pyramidal cells are affected by chloride-cotransport antagonism.

It was noted that the latency between spontaneous discharges of areas CA1 and CA3 significantly increased with exposure to low-[Cl\(^{-}\)]\(_o\) medium. As well, there was a significant increase in the latency between electrical stimulation of the Schaffer collaterals and the consequent CA1 epileptiform burst discharge. These latencies increased from a few milliseconds before low-[Cl\(^{-}\)]\(_o\) exposure, to as much as 30–40 ms after prolonged low-[Cl\(^{-}\)]\(_o\) exposure, just before the cessation of bursting in area CA1. It is unlikely that the long latencies between the activation of the presynaptic fibers and CA1 epileptiform discharge were due to changes in synaptic transmission, because even after the CA1 field response was blocked completely by prolonged exposure to low-[Cl\(^{-}\)]\(_o\) medium, CA1 pyramidal cells were found that would discharge action potentials at short latency to Schaffer stimulation. However, prolonged exposure to low-[Cl\(^{-}\)]\(_o\) medium did induce an increase in the latency between Schaffer stimulation and...
part of the antidromically evoked hyperexcitable burst discharge in area CA3. In this case, there were two distinct components to the CA3 response—an antidromic population spike that showed no significant increase in latency and a burst discharge the latency of which increased with exposure time to low-\([\text{Cl}^-]\) medium until it was blocked completely (Fig. 3). Because the burst discharge that followed the CA3 population spike was likely due to local recurrent excitatory interactions in CA3 (Wong and Traub 1983), this finding suggests that the effects of low-\([\text{Cl}^-]\) exposure on the generation of epileptiform discharges in CA3 (involving local recurrent excitatory connections) were similar to those observed in CA1—although a significantly longer time of exposure to low-\([\text{Cl}^-]\) medium was required to affect the CA3 response.

**Chloride-cotransporter blockade and the fidelity of axonal conduction**

We propose, as a possible explanation of our observations described in the preceding text, that blockade of chloride cotransport results in the failure in the ability of action potentials to invade a subpopulation of axonal arborizations (or varicosities) of both the Schaffer collateral pathway and the local recurrent connections in area CA3. The total number of axonal branches affected, or the degree to which a specific subpopulation is affected, are likely dependent on the time of exposure to treatments that antagonize chloride cotransport. This mechanism would result in a time-dependent reduction in the fidelity of transmission of spontaneous burst discharges from CA3 to CA1 as well as a reduction in the strength of local recurrent excitatory activity in CA3. This explanation is consistent with our conclusion that the major effects of low-\([\text{Cl}^-]\) treatment on area CA1 neuronal activity are not dependent on direct actions at excitatory synapses and cannot be attributed to changes in the electrophysiological properties of either presynaptic or postsynaptic neurons. Assuming that electrical stimulation of the Schaffer collateral pathway activates primary axonal branches necessary for the generation of the antidromic CA3 population spike, our observations suggest that the mechanisms affected by low-\([\text{Cl}^-]\) exposure in the CA3-to-CA1 circuit are likely to be presynaptic with respect to area CA1 but must be subsequent to the primary axonal branches of CA3 pyramidal cells. An effect on a specific, nonvarying subset of axonal structures, distal to the primary branches, could explain the consistent phase lags in action potential firing times observed in CA1. If action potentials were prevented from invading some fraction of the total number of synaptic terminals, the total amount of current delivered to the postsynaptic neurons would be diminished. Further, a reduction in the strength of recurrent excitatory activity in CA3 would result in a smaller number of CA3 neurons firing at a given moment. The consequence of these effects would be a weaker excitatory drive by CA3 onto CA1. We observed, just before the total blockade of the stimulation-evoked CA1 field response, long latencies (>30 ms) between Schaffer collateral stimulation and the CA1 population discharge. It seems unlikely that these significant latencies can be explained on the basis of changes in the monosynaptic drive of CA3 onto CA1. Stimulation of the Schaffer collaterals causes some fraction of the CA1 population to depolarize at short (monosynaptic) latency, but the effect appears to be sufficiently desynchronized so that no field response is detected.

Accumulation of \([\text{K}^+]_o\) has been implicated in numerous systems as an important factor controlling action potential propagation in axons (Adelman and Palti 1969; Grossman et al. 1979; Luscher et al. 1994a,b; Malenka et al. 1981; Nicoll and Alger 1979; Parnas 1972; Parnas et al. 1976; Rang and Ritchie 1968; Smith 1980). In response to a short train of high-frequency stimuli delivered to the parallel fibers in rat cerebellar cortex slices (Malenka et al. 1981), it was found that the latency increased and the amplitude decreased in the postsynaptic field response—an effect paralleling our observations here. The magnitude of these changes in the field responses was found to be correlated tightly to the degree of extracellular potassium accumulation. A study on dorsal root ganglion cells suggested that, under conditions such as repetitive stimulation, failure of action potentials to invade the cell soma occurred at sites of impedance mismatch such as branch points (Luscher et al. 1994b). In cases of action potential failure, the electrotetrogenic responses at the soma to failed action potentials had discrete amplitude levels, suggesting that failures of action potential invasion always occurred at the same sites along the axon. This phenomenon of “deterministic failure” is a possible parallel to our observations that the phase lags between pairs of neurons always are fixed with respect to one another.

A number of other factors undoubtedly are affected by the blockade of chloride-dependent cotransport—although it is unclear to what extent they play a role in the consequent desynchronizing effects. For example, blockade of activity-induced changes in the ECS could lead to a reduction of ephaptic field interactions (Faber and Korn 1989; McBain et al. 1990). Previous optical imaging studies of slices suggested that concomitant with the furosemide-blockade of spontaneous bursting was a blockade of activity-evoked volume changes of the ECS (Hochman et al. 1995), indicating that activity-evoked shrinkage of the ECS is blocked by chloride-cotransport antagonism. This action would discourage ephaptic interactions that occur as ECS resistivity increases. Because field interactions may play an important role in neuronal synchronization (Dudek and Traub 1989), the blockade of such ephaptic effects might contribute to the antiepileptic effects of chloride-cotransport antagonism. Another possible factor affected by chloride-cotransport antagonism includes the changes in pH dynamics of intra- and extracellular spaces, affecting putative pH-dependent signaling mechanisms (Chesler 1990; Gottfried and Chesler 1994; Pappas and Ransom 1994; Ransom 1992). It might be that ionic and pH changes induced by the blockade of chloride cotransport reduce the degree of electrotonic coupling between neurons through gap junctions (MacVicar and Dudek 1981); such coupling is known to be labile and affected by changes in the ionic environment and pH (Church and Baimbridge 1991; Neyton and Trautmann 1985). Recently a novel form of activity-dependent action potential gating in CA3 cells has been proposed where the action potential block requires a hyperpolarizing prepulse to activate an A-type \([\text{K}^+]\) channel (Debanne et al. 1997; Kopysova and Debanne 1998). This type of action potential blockade also might play a role in our study because it has been reported that treatments that cause a reduction of the transmembrane chloride gradient in nonmyelinated mammalian axons can lead to dramatic increases in the magnitude and duration of afterpotential hyperpolarizations (Rang and Ritchie 1968). Both furosemide and low-\([\text{Cl}^-]\) treatments result in a diminished transmembrane chloride gradient in hippocampal and cortical pyramidal cells (Misgeld et
Taken together with the findings of our previous study (Hochman et al. 1999), the present results suggest that ionic fluxes mediated by the glial Na⁺K⁺2Cl⁻ cotransporter directly affect the statistical coherence of action potential firing times in neuronal populations. Our data suggest that antagonism of chloride cotransport can induce phase lags between the action potential firing times of CA1 neurons sufficiently large so that both spontaneous and stimulation-evoked population discharges are blocked even though the individual postsynaptic neurons continue to generate monosynaptic EPSPs (and action potentials) in response to Schaffer stimulation. This incoherence in action potential firing associated with burst discharges might underlie the potent antiepileptic effects of chloride-cotransport antagonism. This effect is “nonsynaptic” because it is not mediated by changes in synaptic transmission per se but rather by changes in the “gating” of action potentials allowed to invade the synaptic terminals of the presynaptic neurons. Given the potency that modulation of chloride cotransport has on population discharge under pathophysiological conditions, it may be that this mechanism also plays a role in the modulation of synchronized activity necessary for normal function. An understanding of how chloride cotransport modulates the coherence of action potential firing might shed light on the contributions of nonsynaptic mechanisms to the generation and maintenance of synchronized activity as well as suggest new strategies for the development of antiepileptic treatments.

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