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ENDOGENOUS AND NETWORK BURSTS INDUCED BY *N*-METHYL-D-ASPARTATE AND MAGNESIUM FREE MEDIUM IN THE CA3 REGION OF THE HIPPOCAMPAL SLICE

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Abstract—The epileptogenic properties of *N*-methyl-D-aspartate and magnesium-free medium were investigated in the CA3 region of the hippocampal slice preparation in the rat. Bath application of *N*-methyl-D-aspartate (5–10 μM) or magnesium-free medium induced both spontaneous and stimulus-evoked bursts. Both endogenous and network bursts were generated, the former always preceding the latter. The paroxysmal depolarizing shift underlying the network bursts generated by *N*-methyl-D-aspartate and magnesium-free medium resembled a giant excitatory postsynaptic potential with a reversal potential near 0 mV and a synaptic input in the apical dendrites above the mossy fibre zone. In the presence of *N*-methyl-D-aspartate or magnesium-free medium, population bursts were synchronized by activating single CA3 neurons. *N*-methyl-D-aspartate receptor antagonists prevented the development of *N*-methyl-D-aspartate-induced spontaneous and stimulus-evoked bursts. However, the only *N*-methyl-D-aspartate receptor antagonist effective in preventing such bursts in magnesium-free medium was DL-3-[(±)-2-carboxypiperazin-4-yl-]-propyl-1-phosphonic acid.

Endogenous bursting in the CA3 region has not been observed with other convulsants and thus may reflect the novel voltage dependence of the *N*-methyl-D-aspartate receptor gated ionic channel. *N*-methyl-D-aspartate receptors may also partially contribute to the excitatory interaction between CA3 neurons and thereby account for the synchronization of the population observed when activating single CA3 neurons.

Interest in the role of *N*-methyl-D-aspartate (NMDA) receptors in the genesis of hippocampal epileptiform activity has grown since the demonstration that selective NMDA antagonists reduce such activity in the CA1 region.[12] Recently, several groups have reported that bursting develops in the CA3 region when hippocampal slices are superfused with Mg-free medium.[1, 18, 20, 23] It has been suggested that these bursts arise in part as a result of removing the Mg^{2+} which normally blocks the NMDA receptor gated ionic channel.[1, 18, 22, 23]

Superfusion of NMDA also induces bursting in the same region.[2] Despite the widespread interest in the action of NMDA, the mechanism by which these bursts are generated in the CA3 region has not been studied in detail. In the present study the nature of the bursts induced by NMDA and Mg-free medium were examined. A preliminary account of this material has been presented elsewhere.[19]

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Abbreviations: ACSF, artificial cerebrospinal fluid; APV, D-2-amino-5-phosphonovalerate; AP-7, DL-2-amino-7-phosphonoheptanoate; CPP, DL-3-[(±)-2-carboxypiperazin-4-yl-]-1-phosphonic acid; EPSP, excitatory postsynaptic potential; NMDA, *N*-methyl-D-aspartate; PDS, paroxysmal depolarizing shift; PSTH, post stimulus time histogram.

EXPERIMENTAL PROCEDURES

Methods

Hippocampal slices were prepared from Wistar rats (90–200 g) as detailed previously.[9] Briefly, animals were killed by a heavy blow, the brain rapidly removed, the hippocampus dissected free and sliced transversely on a McIllwain tissue chopper at a nominal thickness of 500 μm. Slices were maintained at room temperature in a holding chamber bubbled with 95% O_2/5% CO_2 until required. Recordings were made from submerged slices at a bath temperature of 33–34°C. The artificial cerebrospinal fluid (ACSF) had the following composition (mM): NaCl 126; KCl 3.5; $CaCl_2$ 2; $NaHCO_3$ 25; $NaHPO_4$ 1.2; $MgCl_2$ 1.3; glucose 11. Equilibrating the ACSF with 95% O_2/5% CO_2 gave a pH of 7.3–7.4. Drugs were dissolved in the ACSF and applied via a three-way tap system. The flow (2.75 ml/min) and dead volume were such that the dead time before a drug reached the bath was 30–40 s. Equilibrium was apparently reached within 2–3 min.

Intracellular recordings were made with microelectrodes filled with 4 M K-acetate, 2 M K-methylsulphate, 2 M CsCl or 2 M Cs_2SO_4. Extracellular recordings were made with glass micropipettes filled with 4 M NaCl. Bipolar etched tungsten electrodes or twisted nichrome wires were used for stimulation. Square wave voltage pulses (10–100 μs, 5–150 V) were used for stimulation. The stimulation electrode was placed on the outer edge of the upper blade of the granule cell layer and as such both the mossy fibres and the temporo-ammoniac pathway were stimulated. Stimulation is thus referred to simply as "stimulation". A standard preamplifier with an active bridge (Axoclamp II) was used for intracellular recording and current injection. Recordings from the intracellular electrode and field potential recordings from the extracellular electrode were displayed on a Nicolet 3091 digital oscilloscope. From the oscilloscope they were sent to a computer for storage and from there at a reduced rate to a Brush recorder.

Statistical comparisons between means were made using the t-test. Post stimulus time histograms were tested for significance using the chi-square test. It was assumed that, in the absence of stimulation, the expected values would be equally distributed across intervals. Data was judged to be significant when $P < 0.05$. Averaged data are presented as $\bar{X} \pm$ S.D.

Materials

The drugs used were: DL-2-amino-7-phosphonoheptanoate (AP-7; Sandoz); D-2-amino-5-phosphonovalerate (APV; Cambridge Research Biochemicals); DL-3-[(±)-2-carboxy-piperazin-4-yl-]-propyl-1-phosphonic acid (CPP; Sandoz); *N*-methyl-D-aspartate (NMDA; Cambridge Research Biochemicals). Drugs were dissolved in water and kept frozen until used at which time they were diluted further. Mg-free ACSF was made by eliminating $MgCl_2$. From the analysis on the chemical labels (Merck), Mg-free ACSF contained less than 5 μM Mg^{2+}.

RESULTS

The observations reported are based on intracellular recordings from 42 CA3 neurons with a membrane potential greater than −57 mV and with action potentials greater than 70 mV. Apparent input resistance, measured with hyperpolarizing pulses, was 77 ± 27 MΩ (range 26–125 MΩ). In 11 slices only extracellular recordings were made.

N-Methyl-D-aspartate and magnesium-free medium generate network type bursts

Two types of bursts have been identified in CA3 neurons, network and endogenous.[11,13] Using the criteria of Johnston and Brown,[13] we examined the nature of the bursts induced by NMDA and Mg-free ACSF.

Network bursts represent a population response and as such: (1) each intracellular burst should be accompanied by an extracellular field potential reflecting the synchronous activation of the population; (2) evoked bursts should be variable in latency and strongly dependent on stimulus intensity, reflecting the polysynaptic nature of the network; (3) burst frequency should be independent of membrane potential. As shown in Fig. 1, the bursts induced by NMDA and Mg-free ACSF meet these criteria.

If the PDS is generated as the result of a network event resembling an excitatory postsynaptic potential (EPSP)[14] then it should be a monotonic function of membrane potential. This was tested by shifting the membrane potential with current injection and determining the amplitude of the PDS. Depolarizing the membrane reduced the amplitude of the PDS, induced by NMDA or Mg-free ACSF, until it finally reversed and became negative (Fig. 2). The reversal potential for both NMDA and Mg-free ACSF was near 0 mV (2 ± 3.5 mV and 0.0 ± 1.3 mV respectively; $n = 3$), a value similar to that reported for picrotoxin,[13] kainate[5] and mast cell degranulating peptide[7] induced bursts.

Dendritic site of burst generation

As the bursts induced by NMDA and Mg-free ACSF were network events, there should be one or

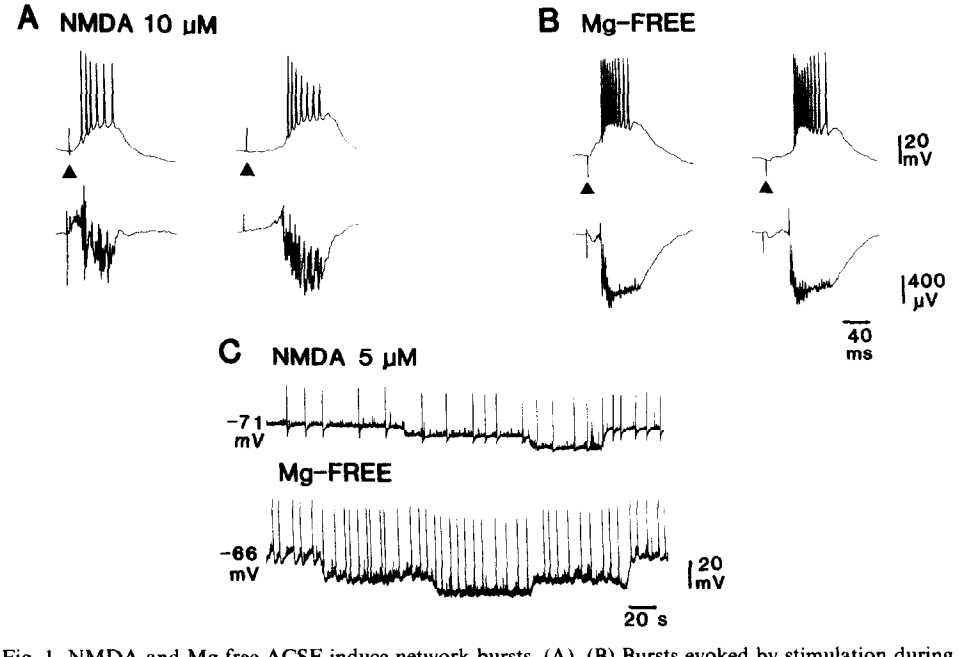

Fig. 1. NMDA and Mg-free ACSF induce network bursts. (A), (B) Bursts evoked by stimulation during superfusion with NMDA (10 μM) or Mg-free ACSF. The upper and lower tracings are intracellular and extracellular recordings respectively. Note that both the form and time course of the PDS were similar to the corresponding events in the field recordings. In each set of tracings, the stimulus intensity on the right was 20% of that on the left. Despite the decrease in stimulus intensity the form of the bursts was not altered although the latencies were increased. (C) Hyperpolarizing the membrane by means of current injection did not abolish or reduce the bursts induced by NMDA or Mg-free ACSF.

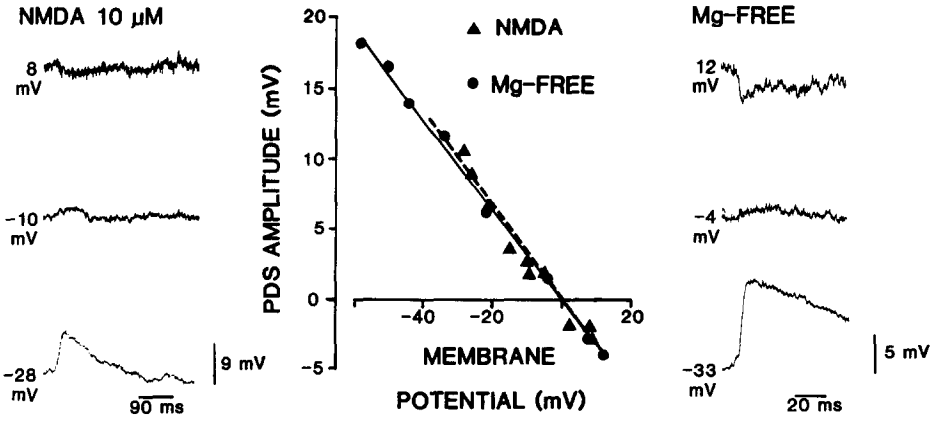

Fig. 2. The amplitude of the PDS induced by NMDA and Mg-free ACSF varies monotonically with membrane potential. The amplitude of the PDS in NMDA and Mg-free ACSF from two different neurons is plotted as a function of the membrane potential. The broken and solid lines were obtained by least squares fit of the NMDA and Mg-free PDS amplitude data. Note the similarity of the reversal potentials. Recording electrodes were filled with Cs_2SO_4 to reduce potassium currents.[5]

more sites along the dendritic tree at which the giant EPSPS are produced which account for the PDS.[3,14] A laminar profile analysis was therefore made to determine the location of this site or sites. As shown in Fig. 3, at the level of the cell body layer and below, the bursts recorded in the field electrodes were positive. The positive deflection suggests these are sources for current sinks located elsewhere.[17,24] The polarity of the field potential began to reverse in the mossy fibre region, reaching the maximum negativity in the apical dendrites above the mossy fibre region (Fig. 3). As the negativity corresponds to the sink region, this analysis suggests that the synaptic inputs generating the bursts are located in the apical dendrites. This distribution is in keeping with what has been found for the bursts induced by kainate, D-tubocurare and elevated K^+.[4,5]

N-*Methyl*-D-*aspartate and magnesium-free medium also generate endogenous bursts*

In six out of 18 neurons NMDA (5–10 μM) was observed to first generate endogenous bursts (Fig. 4), i.e. intracellularly recorded bursts without a concom-

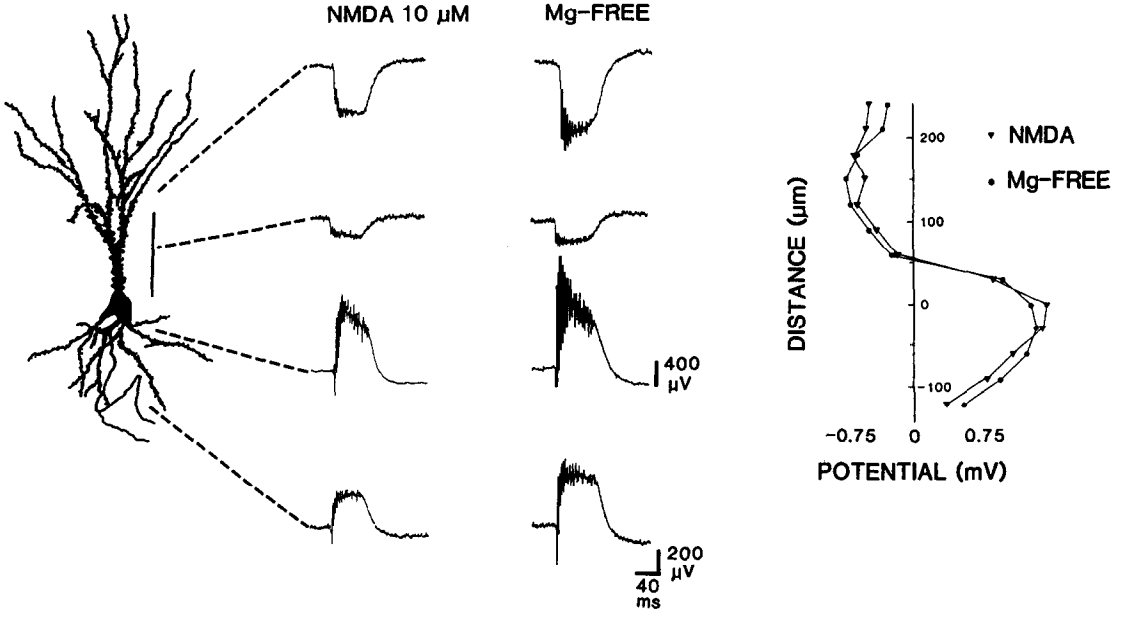

Fig. 3. Laminar profile of spontaneous bursts induced by NMDA and Mg-free ACSF. On the left is a camera lucida drawing of a Golgi-stained CA3 neuron for reference (calibration bar = 100 μm). The dashed lines indicate the locations from which the representaive field potentials were recorded. Note that at the level of the pyramidal cell layer and below the bursts were positive whereas above this region they gradually became negative. On the right is plotted the burst amplitude as a function of distance from the pyramidal cell layer (0 μm) for the same slice from which the tracings were obtained. Note that the peak negativity of the bursts is located between 125 and 175 μm above the pyramidal cell layer.

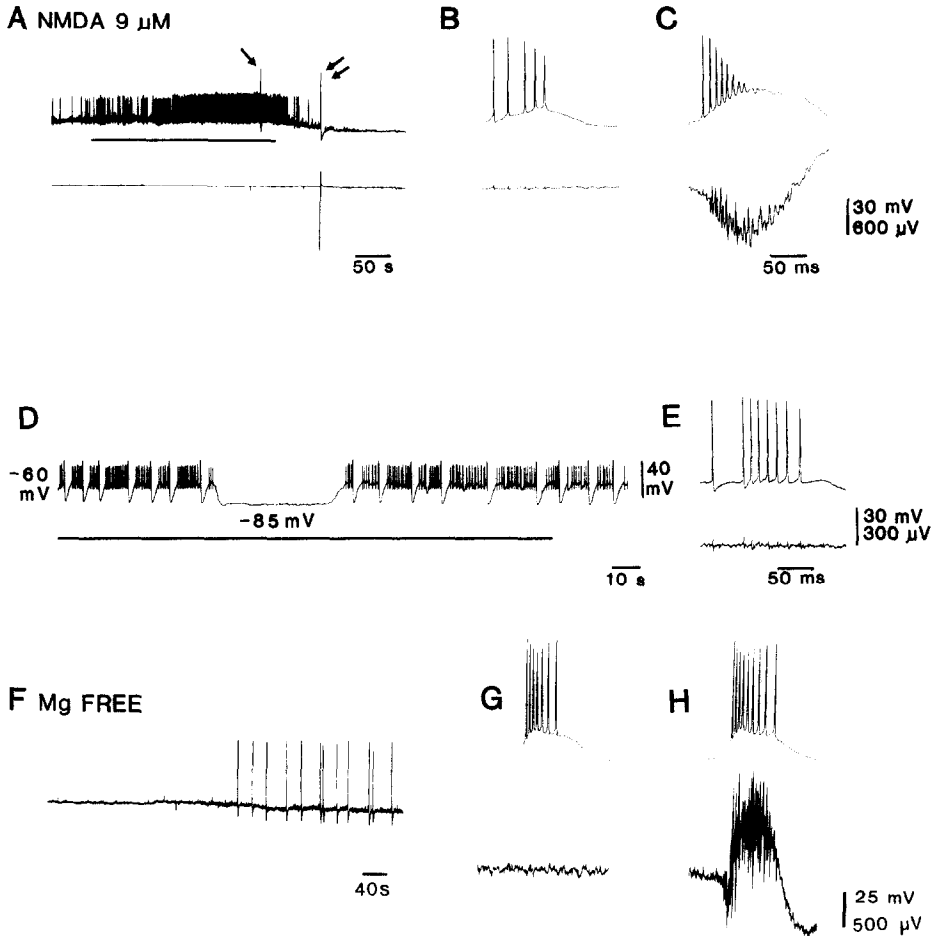

Fig. 4. NMDA and Mg-free ACSF induce endogenous bursts. (A) Application of NMDA resulted in two bursts (indicated by arrows). Note that only the second burst was accompanied by a population potential in the field recording (lower trace). (B), (C) Records of the first and second bursts respectively at a faster sweep. Note the absence of a population response in (B) compared to that in (C). (D) Application of NMDA (10 μM) induced spontaneous bursts, one of which is shown at a faster sweep in (E). Note the absence of a burst in the extracellular field recording. Bursts were reversibly abolished by hyperpolarizing the membrane (D). Compare this record with the traces in Fig. 1C. (F) Application of Mg-free ACSF first produced an endogenous burst (G) and then network bursts as in (H).

itant population response. In three neurons, NMDA (5 or 10 μM) induced a long enough series of bursts without population responses that the effect of membrane polarization on the bursts could be studied (Fig. 4D). Mg-free ACSF also induced endogenous bursts (Fig. 4; $n = 3$).

Synchronization of bursting by single cell activation

Since endogenous bursts were recorded in a number of cells before network bursts developed, we wondered if in fact neurons firing in this manner actually triggered the network bursts as the excitability of the tissue was increased.

Our experiments were similar in design to those described by Miles and Wong.[15] The microelectrodes used to record from and activate the single neurons were filled with 2 M Cs_2SO_4 or CsCl to permit ready generation of bursts of action potentials with injected pulses of current.[6] The membrane potential was held hyperpolarized by steady current injection to ensure that spontaneous activity from the cell was abolished and only activity evoked by current injection or that arising from population events (bursts) was present in the traces.

Figure 5A illustrates one experiment with NMDA. The mean frequency of bursting in NMDA was 0.065 Hz. When the cell was stimulated at a frequency of 0.29 Hz with current pulses of sufficient intensity to generate 10–15 action potentials, the frequency of bursting was significantly increased to 0.105 Hz (62% increase in burst frequency). The effect of the stimulation was to shift the distribution of interburst intervals to the left. In two out of three neurons tested in this manner, single cell stimulation increased the frequency of bursting.

Essentially similar results to those with NMDA were observed in the presence of Mg-free ACSF in five out of six neurons. Single cell stimulation increased

the burst frequency from 15 to 34% above the control frequency. As with NMDA, stimulation shifted the distribution of interburst intervals to the left (Fig. 5B). It should be noted that on return to control ACSF, single cell stimulation continued to evoke action potentials in the cell under study but this no longer resulted in network bursts.

Figure 5C is a trace showing the development of synchronization. The post stimulus time histogram (PSTH) of this experiment is shown in Fig. 5D. The average frequency of this series (0.38 Hz) was significantly different from the control (0.29 Hz) and the PSTH was significantly different from that expected by chance.

Effects of N-*methyl*-D-*aspartate antagonists on the bursts induced by* N-*methyl*-D-*aspartate and magnesium-free medium*

In agreement with Anderson *et al.*,[2] superfusion of the NMDA antagonists (APV, AP-7 or CPP; 10–30 μM; $n = 6$) reduced or prevented the bursting induced by 10 μM NMDA. However, with the exception of CPP (15–30 μM; $n = 3$ out of 4) the NMDA antagonists (APV, AP-7; 30–50 μM; $n = 12$) failed to prevent the development of spontaneous or evoked bursts in Mg-free ACSF, although the bursts were typically shorter in duration (Fig. 6). This apparent inconsistency between CPP and the other NMDA antagonists may result from CPP binding to both benzodiazepine and NMDA receptors.[25]

DISCUSSION

In the present report we have shown that both NMDA and Mg-free ACSF can induce network and endogenous bursts in CA3 neurons. Single cell stimulation in the presence of NMDA or Mg-free ACSF can partially synchronize a population of neurons. Furthermore, it is clear from our observations with APV and AP-7 that the bursts induced by Mg-free ACSF are only partially dependent on an interaction with NMDA receptor gated ionic channels (see also Refs 18 and 21 for discussion on NMDA receptors and bursts in the CA3 region).

Network bursts

Our observations that NMDA and Mg-free ACSF induce spontaneous and stimulus-evoked

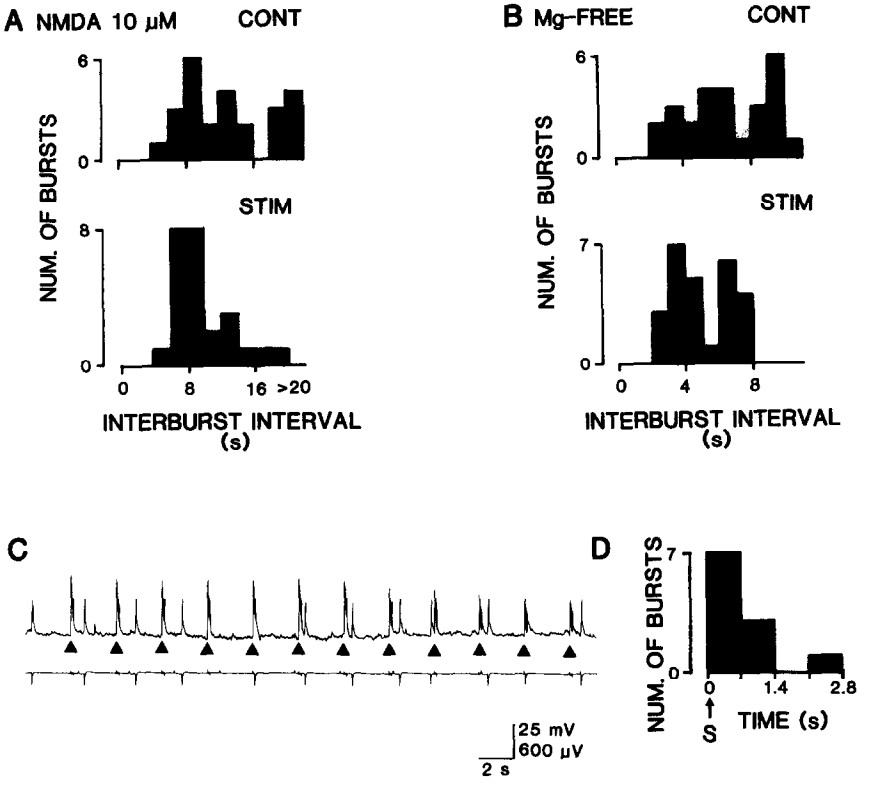

Fig. 5. Single cell stimulation in the presence of NMDA or Mg-free ACSF increases the frequency of spontaneous bursts and partially entrains bursts. (A), (B) Distribution of interburst intervals in NMDA and Mg-free ACSF before (cont) and during single cell stimulation (stim). Stimulation significantly increased burst frequency in both (A) and (B). (C) Records from another neuron superfused with Mg-free ACSF. Upper and lower traces are intracellular and extracellular recordings respectively. Current was injected through the intracellular micropipette at times indicated by the triangles. Bursts, but not current injection, were accompanied by concomitant field potentials. Note that by the fifth stimulus the bursts tended to occur shortly after the stimulus. (D) Post stimulus time histogram of the data shown in (C).

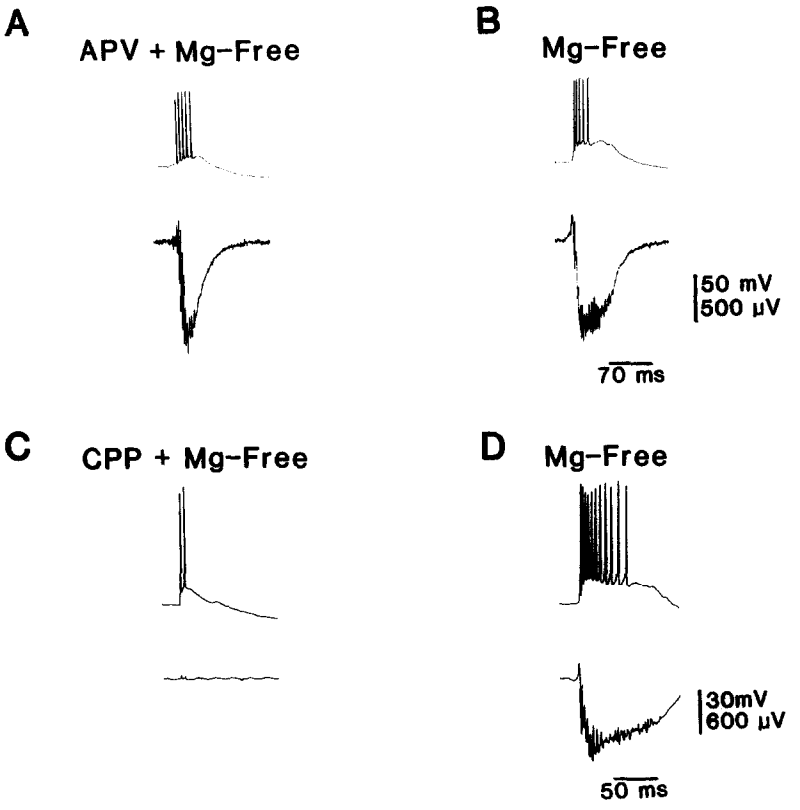

Fig. 6. CPP, but not APV, prevents the development of bursts in Mg-free ACSF. Upper and lower traces are simultaneous intracellular and extracellular recordings respectively. Superfusion of APV (50 μM) prior to (10 min) and continuous with Mg-free ACSF did not prevent the development of bursts. (A) Spontaneous burst recorded 4 min after addition of Mg-free ACSF plus APV. (B) Control response recorded in Mg-free ACSF after washout of APV. Note the increase in burst duration compared with (A). (C) Experiment as in (A) with another neuron but employing CPP (20 μM) as the antagonist. Mg-free ACSF in the presence of CPP induced two spikes but no bursts. Note lack of population response in field recording. (D) Control response in Mg-free ACSF after washing CPP.

bursts in CA3 neurons are in agreement with previous studies.[1,2,18,20,23] The bursts are activated by extrinsic mechanisms, most likely synaptic, and the underlying PDS has properties resembling a giant EPSP.[3,13,14] These network bursts closely resemble those induced by kainate and other convulsants.[4,5,13] Thus, the laminar profile analysis of the spontaneous bursts induced by NMDA and Mg-free ACSF revealed that the maximum negativity was in the apical dendrites above the mossy fibre zone. It is in this region that excitatory collaterals from CA3 neurons are thought to make synaptic contact with each other and may contribute to burst generation.[16] This is also the region of maximum negativity observed with bursts induced by kainate. The reversal potential for the bursts induced by NMDA and Mg-free medium are also similar to the reversal potential for other convulsants. Taken together these observations suggest that there is likely to be a common mechanism generating network bursts, even though the bursts may be initiated in different ways.

Endogenous bursts

Endogenous burst activity induced by NMDA or Mg-free ACSF has not been previously described in the CA3 region of the hippocampus. Other convulsive agents or procedures applied to the CA3 region including kainate, D-tubocurare, mast cell degranulating peptide and elevated K^+, do not produce such bursts.[4,5,7] Endogenous bursts may be initiated by the unique interaction of Mg^{2+} with the NMDA receptor gated ionic channel[22] as demonstrated in cortical neurons[8] and lamprey spinal neurons.[10]

Burst synchronization

The synchronization of bursts by single cell stimulation in NMDA and Mg-free medium is similar to that observed in the disinhibited slice,[15] in which the excitatory interaction between CA3 neurons is enhanced leading to synchronization.[16] The excitatory collaterals between CA3 neurons may, to some extent, utilize NMDA receptors. In the presence of NMDA

or reduced concentrations of MG^{2+}, the level of excitability may be raised sufficiently such that activating one CA3 neuron eventually leads to synchronization of the population.

Whatever the mechanism, population synchronization by single cell stimulation implies that a special pacemaker region may not be required for the generation of bursts. Instead, the neurons with the lowest threshold may serve as pacemakers for the population as a whole. The fact that the CA3 region can be cut into a small section, isolated from the rest of the hippocampus, ans still generate bursts, as for example when disinhibited,[15] is consistent with such an interpretation.

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