Intrinsic NMDA-Induced Oscillations in Motoneurons of an Adult Vertebrate Spinal Cord Are Masked by Inhibition

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Rioup-Ledotti, Mengia-Seraina. Intrinsic NMDA induced oscillations in motoneurons of an adult vertebrate spinal cord are masked by inhibition. J. Neurophysiol. 77: 717–730, 1997. Low-frequency membrane potential oscillations were induced in motoneurons (MNs) of isolated hemisected frog spinal cords during N-methyl-d-aspartate (NMDA) application. Oscillations required the presence of physiological Mg\(^{2+}\) and preincubation with strychnine, whereas incubation with bicuculline or phaclofen was not effective. Oscillations were evident in intracellular recordings from single MNs and simultaneous extracellular recordings from lumbar ventral roots. In Mg\(^{2+}\)-free solution, MNs exhibited irregular transient membrane potential depolarizations that were blocked by D,L-2-amino-5-phosphonopentanoic acid (APV) but not by 6-cyano-7-nitroquinolinoxaline-2,3-dione (CNQX). Generation and maintenance of membrane potential oscillations required specific NMDA receptor activation. Oscillations were antagonized by APV but not by CNQX. Strychnine preincubation was required for NMDA to induce oscillations, but was not critical in maintaining them, because oscillations persisted after removal of strychnine. Therefore oscillations are suggested to be an inherent property of the spinal neuronal circuitry. Tetrodotoxin (TTX) blocked spike activity and had a bimodal effect on membrane potential oscillations. Oscillations initially were blocked by TTX, but reappeared spontaneously after 10–40 min. This suggests that maintenance of oscillations, once evoked, does not involve MN firing. Na\(^+\) entry through TTX-insensitive Na\(^+\) channels and/or NMDA receptor channels, transmembrane Ca\(^{2+}\) flux, Ca\(^{2+}\) release from intracellular stores, and Ca\(^{2+}\) activated K\(^+\) channels were critical in controlling the amplitude and frequency of membrane potential oscillations. It is hypothesized that these unmasked intrinsic oscillations in adult frog spinal cord MNs may represent a premetamorphic spinal oscillator involved in tadpole swimming that becomes suppressed during metamorphosis as strychnine-sensitive inhibition becomes more pronounced.

INTRODUCTION

Rhythmic motor output is produced by networks consisting of many synaptically interacting neurons and underlies motor behaviors like locomotion, swallowing, and respiration. In general, rhythmic motor output may result from synaptic interactions alone or from intrinsic membrane properties of neurons within the network, or it may be controlled by a combined modulation of network properties and intrinsic oscillatory properties of neurons. The neuronal mechanisms underlying rhythmic motor activity in vertebrates produced by networks have been shown by Roberts et al. (1986) for swimming in tadpoles, and by Kudo and Yamada (1987) for initiation of locomotor rhythms in the neonatal rat, and reviewed by Grillner et al. (1991) for locomotion in the lamprey. Intrinsic oscillatory properties have been suggested to generate rhythmic motor behavior in spinal cords of the lamprey (Sigvard et al. 1985; Wallén and Grillner 1985, 1987), tadpoles (Sillar and Simmers 1994), and neonatal rats (Hochman et al. 1994), and in brain stem neurons of various vertebrates (swallowing: Tell and Jean 1991, 1993; respiration: Dekin and Haddad 1990; Funk et al. 1993; locomotion: Durand 1993; Orlovsky 1972; Serafin et al. 1992).

Natural rhythmic motor output can be initiated by sensory afferents (e.g., Alford and Williams 1989; Soffe and Roberts 1982a,b) but can also be mimicked by pharmacological treatment with excitatory amino acid agonists. In particular, specific activation of the N-methyl-d-aspartate (NMDA) subtype of glutamate receptors has been shown to induce both rhythmic motor activity (Cazalets et al. 1992, 1994; Dale and Roberts 1984; Hernández et al. 1991; Kudo and Yamada 1987; Roberts et al. 1986; Smith et al. 1988) and membrane potential oscillations in individual neurons (Durand 1991, 1993; Hochman et al. 1994; Serafin et al. 1992; Sillar and Simmers 1994; Tell and Jean 1991, 1993; Wallén and Grillner 1987). In the isolated lamprey spinal cord, bath application of NMDA induces fictive locomotion (Brodin et al. 1985; Grillner et al. 1981; Wallén and Grillner 1987) as well as inherent oscillatory activity in synaptically isolated neurons within the locomotor central pattern generator (Wallén and Grillner 1987). Moreover, NMDA receptor antagonists are able to block naturally induced locomotor activity, indicating that NMDA receptors may play an important role in rhythmic activities underlying naturally occurring locomotion (Alford and Williams 1989; Wallén and Grillner 1987).

The voltage dependence of the NMDA receptor in the presence of physiological levels of Mg\(^{2+}\) causes a negative slope conductance in the current-voltage relationship (Mayer and Westbrook 1987; Mayer et al. 1984; Nowak et al. 1984) and may give neurons carrying NMDA receptors special bistable properties (Wallén and Grillner 1987). These unique properties make the activation of NMDA receptors particularly well suited to produce membrane potential oscillations. It has been shown that similar, but faster locomotor motoneurons may represent a premetamorphic spinal oscillator involved in tadpole swimming that becomes suppressed during metamorphosis as strychnine-sensitive inhibition becomes more pronounced.

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Although found in the developing spinal cord (Hochman et al. 1994; Siller and Simmers 1994), NMDA-receptor-induced oscillations have never been identified in adult vertebrate spinal cords, except in the lamprey, a primitive cyclostome (Sigvardt et al. 1985; Wallén and Grillner 1987). The present experiments demonstrate that motoneurons (MNs) in isolated adult frog spinal cords do not normally show membrane potential oscillations. This is consistent with the nonrhythmic episodic locomotion of adult frogs. However, present data demonstrate that NMDA-induced membrane potential oscillations emerge in the adult frog spinal cord after strychnine-sensitive inhibition is blocked. These synchronized rhythmic depolarizations persist when action-potential-mediated synaptic activity is blocked, and also when strychnine is removed. Maintenance of these oscillations requires Ca\(^{2+}\) and Na\(^+\) flux through NMDA receptor channels and functional intracellular Ca\(^{2+}\) stores to regulate the Ca\(^{2+}\) homeostasis accompanying the NMDA-induced membrane potential oscillations. Some of these data have already been published in abstract form (Rioult-Pedotti 1993).

**METHODS**

Experiments were carried out on isolated, sagitally hemisected spinal cords of adult frogs (*Rana esculenta* and *Rana pipiens*). Animals were cooled on ice until immobilized, then decapitated, and a laminectomy was performed to remove the spinal cord while preserving dorsal roots (DRs) and ventral roots (VRs). The spinal cord was hemisected longitudinally to preserve propriospinal pathways and to permit access of electrodes and drugs that are limited by the pia-arachnoid. DR-VR potentials of the last two lumbar root pairs (lumbar IX and X) were recorded as a control. These segments were selected because they carry most of the hindlimb afferent and efferent fibers. Spinal cords stayed viable for up to 10 days when stored at 4°C, as shown by the stability of DR-VR potentials over time. Hemisected spinal cords were transferred into a temperature-controlled recording chamber with a black Sylgard (Corning) bottom. The spinal cord was fitted (cut surface upward) into a small groove made in the Sylgard and pinned down at its rostral end. Hemisected cords were continuously superfused with oxygenated (95% O\(_2\)-5% CO\(_2\)) Ringer solution containing (in mM) 112 NaCl, 2 KCl, 20 NaHCO\(_3\), 2.8 CaCl\(_2\), 1 MgCl\(_2\), and 17 glucose, pH adjusted to 7.2–7.4. MN oscillations have been studied in 31 hemisected cords (23 *R. esculenta*, 8 *R. pipiens*); single hemisected cords were often used more than once, with several days between competitive NMDA receptor antagonist D,L-2-amino-5-phosphonopentanoic acid (APV) (100 μM) (Fig. 1, A, top, and B, top, traces labeled Control). EPSP amplitude did not change, but the duration increased from 8–11 ms to 0.1–14 s. The duration of synaptic potentials was measured as the time the EPSP took to depolarize evoked multiple spiking on a prolonged depolarizing potential (Fig. 1, B, top, trace labeled Control). If Mg\(^{2+}\) was omitted, DR stimulation evoked multiple spiking on a prolonged depolarizing potential (Fig. 1, B, top, trace labeled Control). EPSP amplitude did not change, but the duration increased from 8–11 ms to 0.1–14 s. The duration of synaptic potentials was measured as the time the EPSP took to depolarize evoked multiple spiking on a prolonged depolarizing potential (Fig. 1, B, top, trace labeled Control). EPSP amplitude did not change, but the duration increased from 8–11 ms to 0.1–14 s. The duration of synaptic potentials was measured as the time the EPSP took to depolarize evoked multiple spiking on a prolonged depolarizing potential (Fig. 1, B, top, trace labeled Control). EPSP amplitude did not change, but the duration increased from 8–11 ms to 0.1–14 s.

**RESULTS**

**Induction of irregular transient membrane potential depolarizations**

Excitatory synaptic transmission between DR afferents and MNs in the frog spinal cord were studied by recording intracellularly from lumbar MNs and extracellularly from VRs. MNs were synaptically activated by electrical stimulation of lumbar DR IX or X and identified as MNs by antidromic activation from the VR. Supramaximal DR stimulation in physiological Mg\(^{2+}\) (1 mM) evoked composite EPSPs having amplitudes of 30–40 mV that usually triggered APs (Fig. 1A, top, trace labeled Control). If Mg\(^{2+}\) was omitted, DR stimulation evoked multiple spiking on a prolonged depolarizing potential (Fig. 1B, top, trace labeled Control). EPSP amplitude did not change, but the duration increased from 8–11 ms to 0.1–14 s. The duration of synaptic potentials was measured as the time the EPSP took to depolarize evoked multiple spiking on a prolonged depolarizing potential (Fig. 1B, top, trace labeled Control). EPSP amplitude did not change, but the duration increased from 8–11 ms to 0.1–14 s. The duration of synaptic potentials was measured as the time the EPSP took to depolarize evoked multiple spiking on a prolonged depolarizing potential (Fig. 1B, top, trace labeled Control). EPSP amplitude did not change, but the duration increased from 8–11 ms to 0.1–14 s.

Substances to be tested were dissolved in Ringer solution and bath applied. Because drug effects developed slowly (up to 1 h) and recovery during washout could take up to 2–7 h, stable recording conditions were required for long time periods. Transmitter agonists, antagonists, and ruthenium red were obtained from Research Biochemicals; caffeine, tetraethylammonium (TEA), bis-(α-aminophenoxy)-N,N,N′,N′-tetraacetic acid (BAPTA), and cholin chloride from Sigma; tetrodotoxin (TTX) from Calbiochem; and dantrolene from Alomone Labs.

Cut DRs and VRs of the last two lumbar spinal cord segments were drawn into bipolar glass suction electrodes for stimulation and recording. Responses to DR stimulation were recorded from VRs (DR-VR potentials, DC coupled recordings, otherwise mentioned in figure legends) and simultaneously from single MNs with conventional microelectrodes. Microelectrodes were filled with 1 M potassium citrate and had resistances between 30 and 80 MΩ. MNs were approached by monitoring the extracellular field potential to antidromic VR stimulation while the microelectrode was lowered stepwise (1 μm) into the ventral horn with an inchworm motor positioning system (Burleigh). The evoked negative field potential increased its amplitude when a MN was close and emerged as an antidromic action potential (AP) as soon as a MN was impaled. Antidromic APs allowed the functional identification of MNs. Rheobase and input membrane resistance were assessed by stepwise current injection (±0.2-μA steps) through the recording microelectrode. Membrane resistance ranged from 2 to 60 MΩ. For experiments in which oscillations were studied and long recording sessions were required, only cells with low membrane resistance (20–60 MΩ) were considered for analysis, because they were stable over longer times than those with high membrane resistance (20–60 MΩ). Stable intracellular recordings could be obtained from the same cell for 4–18 h.

Differentially amplified VR responses (DC coupled, otherwise mentioned in figure legends, low-pass filtered at 3 kHz) and intracellularly recorded responses were stored on a digital tape recorder. Data were either plotted on a chart recorder or recorded off-line from the digital tape recorder with a CED 1401 interface on a PC-AT with the use of Sigavg signal averager software from CED. Data were transferred from the PC-AT to a Sun Spark workstation, stored on a digital data storage (DDS) tape, and analyzed with Sigavg and/or Spike2 from CED and with Excel.
both non-NMDA and NMDA receptor activity contributes to the synaptic transmission between DR afferents and MNs in the lumbar spinal cords of adult frogs.

In normal (1 mM Mg$^{2+}$) Ringer solution and in the absence of DR stimulation, MNs showed spontaneous EPSPs that occasionally reached threshold and triggered an AP. However, in nominally Mg$^{2+}$-free solution these same neurons exhibited irregular spontaneous synaptic potentials that lasted longer, were larger, and more often triggered bursts of APs compared with Mg$^{2+}$-containing solutions (Fig. 2A). Most of these irregular spontaneous depolarizations were abolished in the presence of APV but not CNQX, presuming the particular involvement of NMDA receptor activity. Under Mg$^{2+}$-free conditions, DR stimulation evoked prolonged and synchronized initial or primary bursts (Fig. 2B, long downward arrows) and a series of secondary bursts (Fig. 2B, short downward arrows) or afterdischarges (as described by Traub and Miles 1992) (Fig. 2B) that disappeared in the presence of Mg$^{2+}$ or APV but not CNQX, again supporting the notion of NMDA receptor involvement. As suggested for CA3 hippocampal pyramidal cells by Traub and Miles, this effect could be a mixed action of synaptic NMDA receptor activation (primary burst) and activation of an intrinsic oscillator (secondary bursts). Repeated stimulation prolonged the depolarizing plateau. If the membrane remained depolarized for a sufficient amount of time (Fig. 2C) small-amplitude oscillations of 13–15 Hz (Fig. 2C, arrows) replaced the secondary discharges. The small oscillations resembled those generated by changing intracellular Ca$^{2+}$ concentrations (Li et al. 1994). In synchrony with MN depolarizations and spike activity (Fig. 2B, top), VR recording (Fig. 2B, bottom) also showed bursts of spikes. The resolution of VR recordings, representing DC coupled MN depolarizations and APs, was high enough to follow most of the membrane potential changes recorded in single MNs (Fig. 2, compare MN and VR recording). If stimulated at high frequency (interstimulus interval shorter than required duration for the MN to repolarize) in Mg$^{2+}$-free solution, maintained plateaus could irreversibly depolarize the membrane potential and reduce the overall activity. Full recovery of the DR-VR potential did not always occur, possibly because of neurotoxic damage due to excess Ca$^{2+}$ entry through NMDA receptor channels (Abele et al. 1990; Choi 1988; Choi et al. 1988; Coulter et al. 1992; Manev et al. 1989; Scharfmann and Schwartzkroin 1989).

**Induction of membrane potential oscillations**

The spontaneous responses of MNs in the absence of DR stimulation to different concentrations of bath-applied NMDA were examined for the presence of regenerative membrane potential oscillations, as has been described by others for spinal neurons (e.g., Wallen and Grillner 1987). Low concentrations of NMDA (5–12 μM) in physiological Mg$^{2+}$ had no effect on spontaneous activity in MNs, i.e., responses were indistinguishable from control. At concentrations >12 μM (15–200 μM), MNs depolarized from −60 to −80 mV to a membrane potential of −20 to −30 mV, whereas trains of APs that could last for >1 min were fired around the spike threshold potential. The depolarizing NMDA effect at concentrations >12 μM was concentration dependent: the higher the NMDA concentration, the shorter the time to depolarize MNs. Raising the NMDA concentration from 15 to 200 μM reduced the time necessary to depolarize the neurons from ~60 min (at 15 μM) to 10 min (at 200 μM). DR stimulation in the presence of 15–200 μM...
**Fig. 2.** Spontaneous and evoked responses in Mg$^{2+}$-free solution. 

- **A**: spontaneous irregular membrane potential depolarizations that evoked bursts of APs when spike threshold was reached (MN, top trace). Simultaneous recordings from VRs (VR, bottom trace) show synchronized synaptic potentials and APs. 
- **B**: DR stimulation (upward arrows) under the same conditions evoked long-lasting depolarizations in the same MN and VR. Primary bursts are indicated with long downward arrows, secondary bursts with short downward arrows (for explanations, see text). 
- **C**: response to DR stimulation (upward arrow). Note the small-amplitude oscillations at strongly depolarized potentials (downward arrows). Eight stimuli were applied at 30-s interval between B and C. Horizontal bar: 5 s (A and B), 1 s (C). Vertical bars: 40 mV (top trace), 1 mV (bottom trace).

NMDA did not evoke any response either in MNs or in VRs. However, the preparation was still viable, as evidenced by reliable spike responses to antidromic stimulation or current injection. Membrane potential and responses to DR stimulation recovered within 1 h if washed with normal saline and within 30 min in the presence of APV (100 μM). At no concentration did NMDA evoke the regenerative depolarizations demonstrated in other preparations (Durand 1993; Hochman et al. 1994; Serafin et al. 1992; Sillar and Simmers 1994; Tell and Jean 1993; Wallén and Grillner 1987).

This result led to the idea that other spinal cord transmitters or modulators could influence or mask possible NMDA oscillations in MNs. Because serotonin (5-HT) is known to have a modulatory action on spinal neurons, it was tested first. Either spinal cords were preincubated with 5-HT (50–200 μM) before NMDA was added (N = 3), or 5-HT and NMDA were applied together (N = 3). In either case 5-HT failed to induce (see also Hochman et al. 1994) or unmask (but see Sillar and Simmers 1994) oscillations in the spinal MNs.

To examine the possibility that inhibition normally suppresses NMDA-induced regenerative spiking of MNs, different types of inhibition were blocked with specific antagonists. Application of bicuculline (80 μM), phaclofen (40 μM), or strychnine (20 μM) showed similar effects: DR stimulation evoked multiple spiking on a plateau-like depolarization (Fig. 3; strychnine effect, representative also for bicuculline and phaclofen), indicating a strong contribution of inhibitory activity that is involved in shaping evoked responses. No change in spontaneous activity was observed. After preincubation with bicuculline and phaclofen (until response to DR stimulation was stable, i.e., 30–60 min), application of NMDA reversibly depolarized the membrane potential in a pattern similar to that seen in high NMDA (>12 μM). However, when NMDA was added after preincubation with strychnine (30–60 min), spontaneous regenerative membrane depolarizations of 20–40 mV in amplitude were evoked. These slow depolarizations led to spike bursts of 2–30 APs at 30–40 Hz during the plateau phase (Fig. 4). After repolarization, the membrane slowly depolarized again over 10–45 s to a level at which the fast regenerative depolarization began once more. These regular low-frequency oscillations (>10 s per cycle) were in phase with VR potentials. Polarizing the membrane by current injection did not affect the oscillation frequency. The action of NMDA continued throughout its application and in the presence of strychnine. An example of developing oscillatory behavior is illustrated in Fig. 5. The concentrations of Mg$^{2+}$ (1 mM), strychnine (20–40 μM), and NMDA (30–60 μM) were critical to elicit oscillations, and the responses were never seen if strychnine and NMDA were added simultaneously.

**Maintenance of membrane potential oscillations**

The maintenance of membrane potential oscillations did not require the presence of strychnine. Removing strychnine during stable oscillations did not change their characteristics, and they persisted as long as NMDA was present (Fig. 6). Although strychnine was required to induce oscillations, it was not critical in maintaining them. Membrane potential
oscillations also required the presence of physiological Mg\(^{2+}\) levels. Removing Mg\(^{2+}\) during ongoing oscillations depolarized the neuron and reduced the oscillation amplitude and spike activity, and, finally, oscillations ceased. If Mg\(^{2+}\) was omitted before the application of strychnine and NMDA, the neuron never started to oscillate, but rather depolarized to \(-30\) to \(-20\) mV and remained at this potential.

Maintenance of oscillations was critically dependent on NMDA receptor activation. When APV (100 \(\mu M\)) was added to the preparation, NMDA produced irregular oscillations of low amplitude that fell below the threshold level of spike generation. Spontaneous EPSPs and synaptic noise disappeared (Fig. 7A, top trace). Finally, the oscillations ceased entirely, with the membrane potential remaining at the resting level. When APV was removed from the bathing solution, oscillations and burst activity recovered. Synchronized VR potentials (Fig. 7A, bottom trace) showed essentially the same APV and recovery effects. In contrast to APV, CNQX (5–15 \(\mu M\)) did not have any effect on the oscillation amplitude or frequency (Fig. 7B). These data indicate that membrane potential oscillations are due to a specific activation of NMDA receptors.

Addition of TTX (2–4 \(\mu M\)) during ongoing oscillations had the expected effect of blocking spike generation and synaptic noise (Fig. 8). However, an unusual “bimodal” effect on the oscillations was also consistently observed (Fig. 8, Bi–Biv). The normally persistent oscillations (Fig. 8Bi) slowly decreased in amplitude and finally ceased (Fig. 8Bii). However, oscillations recovered eventually (10–40 min; Fig. 8, Bi-iii and Biv) to the original regular and stable frequency, whereas the amplitude, especially of the VR, remained reduced. The VR recording in TTX shown in Fig. 8 is accompanied by the last (Fig. 8Biv) intracellular recording. Burst activity recovered on washout of TTX (as examined by VR recordings the following day). This bimodal effect demonstrates that voltage-dependent Na\(^+\) conductances are important but not critical to the maintenance of membrane potential oscillations. This spontaneous recovery of oscillations in the presence of TTX indicates that oscillations are at least to some degree intrinsic to MNs. It is remarkable that slow depolarizations in VRs, presumably from synaptic potentials, were still present despite the fact that Na\(^+\) spike generation was blocked by TTX.

Although TTX blocked all spike generation, the MNs remained synchronized, indicated by the persisting oscillations observed in VRs. This synchronization might be mediated

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**FIG. 3.** Effect of strychnine incubation on DR evoked EPSPs (MN, top traces) and DR-VR potentials (VR, bottom traces). Whereas only a few APs were evoked in normal saline (A, Control), multiple spiking on a prolonged depolarizing potential (B) was produced on the same stimulation after strychnine incubation for 30 min. Note: strychnine effect is also representative for bicuculline and phaclofen effects. Horizontal bar: 1 s. Vertical bars: 20 mV (top traces), 1 mV (bottom traces). The variable spike amplitudes are due to aliasing.

**FIG. 4.** Regular low-frequency membrane potential oscillations of a single MN (top trace) and synchronized VR oscillations (VR, bottom trace) in the presence of NMDA (50 \(\mu M\)) and strychnine (30 \(\mu M\)). Horizontal bar: 20 s. Vertical bars: 40 mV (top trace), 1 mV (bottom trace).
by electrical transmission through gap junctions (Perrins and Roberts 1995). This possibility and preliminary results regarding electrical coupling between MNs are discussed later.

NMDA oscillations were maintained, although AP-mediated synaptic transmission was blocked by TTX and NMDA was the only exogenous agonist present (Figs. 8, 10, and 12). The exclusion of synaptic transmission by TTX in these experiments makes it most likely that the rhythmic activity of MNs was induced directly by the presence of NMDA in the bath. Because there is no direct evidence for electrical coupling between MNs and interneurons, present data suggest that EPSPs (Fig. 1) were at least in part due to monosynaptic activation of NMDA receptors, as supported by the evidence about colocalization of NMDA and non-NMDA receptors in the spinal cord (Bekkers and Stevens 1989; Jakowec et al. 1995; Konnerth et al. 1990).

Ionic mechanisms

To examine possible ionic mechanisms involved in the maintenance of MN oscillations, TEA (10 mM) was added to the bathing solution during stable oscillations (Fig. 9A, top trace). TEA increased the oscillation frequency and amplitude and the spike activity at depolarized membrane potentials (Fig. 9A, bottom trace). Similar effects were reported by Wallén and Grillner (1985, 1987), who suggested that during membrane potential oscillations TEA-sensitive K\(^{+}\) currents partly counteract the depolarization and help to reach a plateau before the rapid repolarization starts. Removing extracellular Ca\(^{2+}\) and replacing it with an equimolar concentration of Ba\(^{2+}\) blocked burst activity and depolarized the membrane potential by ~20 mV. However, small-amplitude oscillations remained (Fig. 9B). These effects might be caused partly by Ba\(^{2+}\) blocking transmembrane Ca\(^{2+}\) currents and failing to activate Ca\(^{2+}\)-dependent K\(^{+}\) channels. This would lead to an accumulation of intracellular K\(^{+}\), preventing the membrane potential from entirely repolarizing (e.g., Grillner and Wallén 1985; Hagiuwara and Byerly 1981; Meech 1978). The Ca\(^{2+}\) current through ionic and/or receptor-gated membrane channels is also strongly reduced by Ba\(^{2+}\), which may explain the reduction of oscillation amplitude and the disappearance of spike activity.

The replacement of extracellular Ca\(^{2+}\) with Ba\(^{2+}\) blocked the Ca\(^{2+}\) entry into the cells but still allowed Ca\(^{2+}\) to be extruded from intracellular stores. Therefore extracellular Ca\(^{2+}\) was removed by superfusing the cord with Ca\(^{2+}\)-free solution containing BAPTA (1.5 mM), an extracellular Ca\(^{2+}\) chelator, and in the presence of TTX (Fig. 10). Initially, about every other cycle was reduced in amplitude, and finally disappeared, resulting in a decrease in oscillation frequency. This lower frequency was maintained while the positivity of the remaining oscillations was reduced nearly to zero, but a pronounced negativity remained (Fig. 10Aii). These results showed that oscillations can be maintained in the absence of extracellular Ca\(^{2+}\). In neurons, endoplasmic Ca\(^{2+}\) stores bear channels permeant to Ca\(^{2+}\) from within the stores. These channels are coupled to inositoltriphosphate receptors or ryanozine-sensitive receptors (Simpson et al. 1995), and are also activated by high intracellular Ca\(^{2+}\). One function of these stores is to regulate the homeostasis of intracellular Ca\(^{2+}\). Therefore a
possible contribution of Ca\(^{2+}\) released from internal stores to these remaining small-amplitude oscillations was studied. Dantrolene (30 \(\mu\)M), a selective membrane-permeant inhibitor of Ca\(^{2+}\) release from smooth endoplasmic reticulum (Levesque and Atchinson 1988), was added to the Ca\(^{2+}\)-free/BAPTA- and TTX-containing bathing solution. In this condition, the frequency as well as the amplitude markedly decreased (Fig. 10A\textit{iii}), and oscillations could even stop entirely. Oscillations eventually recovered to the initial frequency and amplitude on washout of BAPTA and dantrolene and addition of extracellular Ca\(^{2+}\). Note in Fig. 10B that the displayed recovery had not recovered to control levels. By contrast, oscillation amplitudes recovered entirely on washout after other treatments (Fig. 12, A\textit{iv} and B, left).

\[\text{FIG. 7. Oscillations are antagonized by APV (N = 10) but not by CNQX (N = 10). A: regenerative depolarizations (left) were reduced in amplitude on APV application (1)). When depolarizations fell below threshold, no APs were triggered anymore (middle) and finally oscillations stopped with the membrane potential at resting level (right). Bottom trace: synchronized VR activity. B: R. pipiens, recordings AC coupled. Oscillations and burst activity did not change when CNQX was added. Histograms (bottom) of average amplitudes and cycle intervals before (N = 45) and after (N = 50) CNQX application. Horizontal bars: 20 s. Vertical bars: 30 mV (A, top trace), 1 mV (A, bottom trace and B).}\]

\[\text{FIG. 8. ``Bimodal'' tetrodotoxin (TTX) effect. A: membrane potential oscillations in the presence of NMDA and strychnine (top trace: individual MN, bottom trace: VR). Bi: adding TTX to the bathing solution blocked all spike activity. Bi\textit{ii}: amplitudes started to decrease and finally ceased. Bi\textit{iii}: 30 min later (time was age, i.e., diffusion dependent) regenerative depolarizations developed again, and continued throughout the experiment (Bi\textit{iv}). Similar ``bimodal'' TTX effects were observed in 18 hemisected cords. Horizontal bar: 20 s. Vertical bar: 30 mV (i–iv), 1 mV (bottom trace).}\]
The disappearance of the membrane potential oscillations in the presence of dantrolene suggests a contribution of Ca\(^{2+}\) released from intracellular stores to the maintenance of these oscillations. The following experiments address the question of which Ca\(^{2+}\) stores might be involved. Caffeine, at both 10 and 20 mM, abolished the oscillations as well as the spike activity, and slightly depolarized the membrane potential by \(\sim 5\) mV (\(N = 4\)). Caffeine blocks Ca\(^{2+}\) release from inositoltriphosphate-sensitive Ca\(^{2+}\) stores at \(\geq 10\) mM (Ehrlich et al. 1994), and induces Ca\(^{2+}\) release from ryanodine-sensitive intracellular Ca\(^{2+}\) stores at \(\geq 5\) mM and ultimately their depletion. The depletion of these Ca\(^{2+}\) stores may be responsible for the disappearance of oscillations. Ruthenium red (RuRed, 5 \(\mu\)M, \(N = 4\)), a compound that inhibits ryanodine receptors, initially resulted in a novel small-amplitude oscillation that did not reach spike threshold but showed numerous small depolarizing transients at the depolarized potential (Fig. 11Aii). Thereafter, burst activity disappeared, but the novel cycles were still clearly different from the original ones (Fig. 11Aiii), and it seems that a second rhythm developed. Finally, the oscillations stabilized and remained regular at increased frequency and 10 to 15 mV depolarized membrane potential (Fig. 11Aiv). Replacing extracellular Ca\(^{2+}\) with Ba\(^{2+}\) in the presence of ruthenium red abolished small depolarizing transients at depolarized potential, indicating that this activity might be mediated by transmembrane Ca\(^{2+}\) stores.

**FIG. 9.** Involvement of voltage-dependent K\(^+\) currents and Ca\(^{2+}\)-induced K\(^+\) currents to maintain regenerative depolarizations. Intracellular recordings from 2 different MNs in the presence of NMDA and strychnine (\(A\), top, and \(B\) top). \(A\): application of tetraethylammonium (TEA) (bottom trace) increased oscillation frequency and the activity at depolarized potential (\(N = 3\)). \(B\): replacement of extracellular Ca\(^{2+}\) by Ba\(^{2+}\) reduced oscillation amplitude, abolished spike activity, and depolarized the membrane by 22 mV, from \(-74\) to \(-52\) mV (bottom trace). Hatched line: resting membrane potential (\(N = 5\)). Horizontal bar: 20 s. Vertical bars: 40 mV (\(A\)), 30 mV (\(B\)).

**FIG. 10.** Effects of extracellular Ca\(^{2+}\) removal and dantrolene on oscillations in the presence of TTX and NMDA (\(R.\) pipiens; recordings AC coupled). \(A\): regenerative depolarizations before (\(Ai\)) and after Ca\(^{2+}\) removal from the bathing solution and addition of the Ca\(^{2+}\) chelator bis-(\(\alpha\)-aminophenoxy)-\(N,N,N',N'\)tetraacetic acid (BAPTA) (\(Aii\)). Adding dantrolene to the BAPTA-containing Ca\(^{2+}\)-free solution prolonged cycle interval and reduced amplitude such that almost no oscillations remained (\(Aiii\)). Regenerative depolarizations recovered quickly on washout with NMDA and TTX (see Fig. 12A). \(B\): average amplitudes and cycle intervals with standard deviations of the data shown in \(A\), recovery from data illustrated in Fig. 12Ai. Similar observations were made in 6 hemisected spinal cords. Because recordings were done from VRs only, no conclusions can be drawn about membrane potentials. Note: Small SDs indicate that oscillations were very regular (\(N = 51, 31, 17,\) and 28, from left to right). Horizontal bar: 20 s. Vertical bar: 2 mV.
Ca\(^{2+}\) flux (Fig. 11B), and the second rhythm introduced by ruthenium red disappeared. The caffeine result suggests and the ruthenium red result confirms that ryanodine-sensitive Ca\(^{2+}\) stores are necessary to maintain oscillations. The Ba\(^{2+}\) effect in the presence of ruthenium red was similar to that of Ba\(^{2+}\) alone, indicating that transmembrane Ca\(^{2+}\) flux was involved to control NMDA-induced membrane potential oscillations, as has already been shown above (Fig. 9B).

Transmembrane Na\(^{+}\) currents have been shown to be mainly responsible to maintain membrane potential oscillations in other systems (Johnson et al. 1992; Serafin et al. 1992; Wallén and Grillner 1987). Replacing most of the extracellular Na\(^{+}\) with choline (10 mM NaCl, 102 mM cholinCl\(_2\)) in the presence of NMDA and TTX reversibly and reversibly depolarized entire MNs in the presence of strychnine (Fig. 12). Initially, the oscillation frequency increased while the amplitude was continually reduced and finally ceased (Fig. 12, Aii, Aiii, and B). These experiments demonstrate that Na\(^{+}\) entry, either through TTX-insensitive membrane channels or through NMDA receptor channels, is crucial to maintain oscillations.

**DISCUSSION**

The present results show that NMDA induces membrane potential oscillations in MNs of adult frog spinal cords and that these NMDA-induced oscillations occur after preincubation with strychnine but not with \(\gamma\)-aminobutyric acid-A/B (GABA\(_A\)/GABA\(_B\)) antagonists or 5-HT. No direct link, however, to a motor behavior in the frog can be drawn. Even more because adult frogs do not usually show rhythmic behavior, in contrast to the swimming behavior of their embryos.

Generation of synchronous low-frequency oscillations requires the relief of strychnine-sensitive inhibition and specific activation of NMDA receptors that initiates Mg\(^{2+}\)-dependent membrane potential bistability. These oscillations, once they are evoked, do not require AP-mediated activity or strychnine to continue but do require transmembrane Ca\(^{2+}\) and Na\(^{+}\) flux and intact intracellular Ca\(^{2+}\) mechanisms. The present findings suggest that MN oscillations in the adult frog are maintained by intrinsic properties of the MNs.

**Depolarizing effect of NMDA**

In the adult amphibian spinal cord NMDA alone did not induce rhythmic depolarizations as has been obtained in other vertebrate preparations (Durand 1993; Serafin et al. 1992; Tell and Jean 1993; Wallén and Grillner 1987). NMDA at concentrations >15 \(\mu\)M, however, consistently and reversibly depolarized entire MN pools. Membrane potentials repolarized on NMDA washout and this repolarization was accelerated by APV. However, repolarization never occurred spontaneously during bath-applied NMDA. It is suggested that NMDA receptor activation leads to increased (Murphy et al. 1987) or even excess Ca\(^{2+}\) entry through NMDA receptor channels (Coulter et al. 1992; Garthwaite 1989; Meldrum and Garthwaite 1990; Siman and Card 1988).

**Role of strychnine-sensitive inhibition**

Preincubation with strychnine prevented NMDA depolarization and the combination induced MN oscillations. NMDA induced oscillations only in the presence of strychnine but not phaclofen, bicuculline, and/or 5-HT in all possible combinations. However, the effect of each of these antagonists to DR stimulation was similar in that they produced multiple spiking on prolonged plateau-like depolarizations (Fig. 3). Strychnine is generally thought of as a glycine antagonist but has been shown to antagonize responses to GABA as well (Langdon and Freeman 1987; Schollfield 1982). It is not likely that strychnine had an effect on GA-
Replacing most of the extracellular Na\(^+\) with choline (10 mM NaCl, 102 mM CholineCl) in the presence of NMDA and TTX (R. pipiens, recordings AC coupled), increased the frequency of oscillations and reduced the amplitude continually until oscillations stopped (A). Before (Ai), 5 min (beginning of Aii) and 10 min (end of Aiii) after starting to perfuse with choline. The regenerative depolarization recovered entirely on washout (Aiv). The frequency remained increased in this particular preparation (Control, see Fig. 10Ai). B: average amplitudes (left) and cycle intervals (right) of successive oscillation cycles before, during (start indicated with an upward arrow), and after (downward arrow: 10-min wash) replacement of Na\(^+\) with choline. Data points were taken from A. Similar observations were made in 6 hemisected spinal cords. Horizontal bar: 20 s. Vertical bar: 2 mV.

BAergic inhibition alone, because preincubation with either bicuculline or phaclofen did not enable NMDA to induce membrane potential oscillations. It is, however, possible that there is an occlusion between responses to GABA and glycine, indicating that they may share a population of receptors or channels, as proposed by Lewis and Faber (1993). Alternatively, some GABA and glycine receptors may have common ionophores (Lewis and Faber 1993). From the present data it is not possible to determine whether strychnine acts on glycine, GABA, and/or shared receptors. Strychnine and glycine have also been shown to interact with acetylcholine receptors (Yadid et al. 1993). The possibility of strychnine having an effect on acetylcholine receptors cannot be excluded, because cholinergic agents were not tested in the present experiments. Strychnine has been reported to block voltage-gated delayed-rectifier K\(^+\) channels (Hille 1992; Shapiro 1977; Stockbridge et al. 1992), which repolarize the membrane after an AP. If strychnine blocked delayed rectifiers in present experiments, a marked broadening of APs would be expected. There was, however, no difference in the duration of APs before and after strychnine application (6.6 ms at half-maximal amplitude), suggesting that there was no effect on K\(^+\) channels.

The role of strychnine in rhythmic behavior of spinal neurons in Xenopus embryos and lamprey has been associated with glycinerergic transmission across the spinal midline (Alford and Williams 1989; Cohen and Harris-Warwick 1984; Dale 1985; Kahn 1982; Russel and Wallén 1983; Soffe 1987). This form of glycinerergic inhibition is not likely to explain the present data, because the experiments were carried out on hemisected cords having no contralateral side. Therefore an ipsilateral strychnine-sensitive inhibition is
suggested to be normally activated by DR stimulation in the hemisected cord.

A possible explanation for the strychnine required to obtain NMDA-induced oscillations might be a shift in the current-voltage relationship of the NMDA receptor as proposed by Kim and Chandler (1995). Strychnine would produce an increase in synaptic activity and lead to a downward shift of the current-voltage curve into a region where the negative slope current can allow the membrane potential of MNs to oscillate. However, this possibility seems to be unlikely, because bicuculline or phaclofen, which lead to the same increase in synaptic activity as strychnine (Kim and Chandler 1995), never lead to oscillations (see section about the role of Mg\(^{2+}\) below). Another possibility to explain the strychnine requirement could be inhibition strong enough to cancel excitatory input (Easton and Gordon 1984), though feed-forward inhibition (Alger and Nicoll 1982). In the spinal cord, glutamatergic afferents would project onto dendrites of MNs and interneurons both carrying NMDA receptors. If the interneurons had also inhibitory dendrodendritic synapses onto MNs, both neurons would respond toafferent transmitter release by generating EPSPs, but the EPSPs produced in the interneurons may locally prevent EPSP propagation in the MN through its inhibitory dendrodendritic synapses on the MN.

Role of Mg\(^{2+}\) in generating spontaneous activity and oscillations

Spontaneous depolarizations that often triggered bursts of APs only occurred in the absence of extracellular Mg\(^{2+}\) (Fig. 2A), and were blocked by APV or Mg\(^{2+}\) but not by CNQX (Fig. 7). Reducing extracellular Mg\(^{2+}\) concentration can cause an increase in excitability by facilitating the release of neurotransmitter at nerve terminals (Douglas 1968) and by removing the Mg\(^{2+}\) block from NMDA receptors (Mayer and Westbrook 1987; Nowak et al. 1984). The present data suggest that removing the Mg\(^{2+}\) block from NMDA receptors is sufficient to induce spontaneous depolarizations (but not oscillations) whereas facilitated transmitter release is not, because spontaneous depolarizations in Mg\(^{2+}\)-free Ringer solution were blocked by APV. That NMDA receptor activation seems to be sufficient for generating spontaneous depolarizations is supported by findings in other CNS structures (Abele et al. 1990; Forsythe et al. 1988; Furshpan and Potter 1989; Meldrum and Garthwaite 1990; Robinson et al. 1993; Watson et al. 1989). Physiological Mg\(^{2+}\) concentration was required to induce and maintain membrane potential oscillations. The membrane potential starts to oscillate between two distinct potential levels only in the presence of Mg\(^{2+}\) and NMDA. This could be due to bistable properties of MNs based on the negative slope conductance of the NMDA receptor (Mayer et al. 1984; Nowak et al. 1984). Bistability in MNs did occur either spontaneously or in response to current injection in normal Mg\(^{2+}\) (data not shown) and has been reported in spinal MNs of other preparations (for review see Hounsgaard et al. 1988; Kiehn 1991).

MN synchronization

Synchronization among MNs was maintained although AP-mediated synaptic transmission was blocked by TTX (Fig. 8). Frog spinal MNs are known to be electrically coupled through gap junctions. Therefore it has often been suggested that electrical transmission is responsible for the synchronized motor output. This electrical coupling hypothesis is supported by evidence from the inferior olive (Llinás 1988) and swimming behavior in Xenopus embryos (Perrins and Roberts 1995). Preliminary results to test whether electrical coupling produces synchronization in the frog spinal cord exist. Electrical transmission was blocked with arachidonic acid, which has been shown to uncouple gap junctions without affecting spike activity (Rioult-Pedotti and Clamann 1992; Schmilinsky-Fluri et al. 1990). Arachidonic acid pre-incubation for 10 h did not prevent NMDA oscillations from being induced (N = 2). Several problems, however, could account for possible ineffective uncoupling: arachidonic acid diffusion and absorption into the lipid bilayers are passive processes, and arachidonic acid micelles have the tendency to aggregate and form larger lipid vesicles that do not diffuse easily. Because of these difficulties, more experiments are required before any conclusion can be drawn.

Another hypothesis that could explain the persistence of synchrony in the absence of AP-mediated synaptic activity is entrainment by graded synaptic interactions in a network of normally spiking neurons. In the presence of TTX, transmitter may be released as a graded function of presynaptic EPSPs produced in the interneurons may locally prevent EPSP propagation in the MN through its inhibitory dendrodendritic synapses on the MN.

Ionic mechanisms

In adult frogs, transmembrane Na\(^+\) and Ca\(^{2+}\) currents are critical to maintain rhythmical oscillations (Figs. 10 and 12). To what extent these transmembrane ion fluxes are receptor and/or membrane channel gated is not clear. Oscillations ceased within 5–10 min after replacement of most of extracellular Na\(^+\) by choline (Fig. 12) but were not affected by block either of voltage-dependent Na\(^+\) channels with TTX (Fig. 8) or of Na\(^+\) currents through AMPA receptor channels with CNQX (Fig. 7B). Therefore it is likely that a substantial part of the total Na\(^+\) current is mediated through NMDA receptor channels. This interpretation is strengthened by the general assumption that the depolarizing current following NMDA receptor activation is mainly carried by Na\(^+\) ions (Mayer and Westbrook 1987), and that Na\(^+\) current through NMDA receptor channels dominates the Ca\(^{2+}\) current (MacDermott et al. 1986). The finding that extracellular Na\(^+\) seems to be a major current carrier for oscillations in the frog spinal cord agrees with data from the lamprey spinal cord (Wallén and Grillner 1987) and MNs in the rat vestibular nucleus (Serafin et al. 1992). According to Wallén and Grillner (1987), Na\(^+\) is mainly responsible for the depolarizing phase, and not Ca\(^{2+}\), because its removal had no effect (Grillner and Wallén 1985). This is in contrast to the present experiments, where removal of extracellular Ca\(^{2+}\) reduced the depolarizing phase of extracellularly recorded oscillations by about half (Fig. 10). This residual response suggests
that the contribution of transmembrane currents is smaller for Ca\(^{2+}\) than for Na\(^{+}\). Blockade of Ca\(^{2+}\) release from intracellular stores with dantrolene removed this remaining response (Fig. 10Aiii), which indicates that Ca\(^{2+}\) release from intracellular stores contributes to maintaining oscillations.

The role of intracellular Ca\(^{2+}\) stores is further supported by the caffeine and ruthenium red experiments in which the intracellular release of Ca\(^{2+}\) was altered. Thus the presence of intracellular Ca\(^{2+}\) stores appears to be critical for the repolarization phase. A too-low intracellular Ca\(^{2+}\) concentration may fail to activate Ca\(^{2+}\)-dependent K\(^{+}\) channels and prevent the membrane from being repolarized.

**Hypothesis about the functional significance of oscillations in adult frogs**

On the basis of present data and current knowledge about rhythmic oscillations and locomotor behavior in other species, the following hypothesis is proposed: in the tadpole inherent oscillatory behavior (central pattern generator) would be expressed during rhythmic swimming and become gradually suppressed by increasing inhibition during development from tadpole to adult frog. After metamorphosis, suppressed central pattern generator activity might selectively be relieved and used for walking and swimming. This hypothesis is consistent with the observation that oscillations occur only after disinhibition in the adult frog. There is evidence for the existence of NMDA-evoked rhythmic movements resembling those found during swimming and struggling in *Xenopus* embryos (Dale and Roberts 1985; Soffe 1993). Experiments to test this hypothesis have been carried out and have been presented in abstract form (Rioult-Pedotti 1994).

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