The role of AMPA receptor desensitization and the side effects of a DMSO vehicle on reticulospinal EPSPs and locomotor activity

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ABSTRACT

Activation of the vertebrate locomotor network is mediated via glutamatergic synaptic drive, normally initiated by the brainstem. Previous investigations have studied the role of glutamate receptors, especially NMDA receptors, in generating and regulating locomotor pattern generation. Few studies, however, have focused on the role of AMPA receptors in shaping network activity, especially with regard to their rapid desensitization. It is important to determine if AMPA receptor desensitization plays a role in regulating neuronal network activity. We examined this question on both the network and synaptic levels in the lamprey (*Lampetra fluviatilis*) spinal cord using a selective and potent inhibitor of AMPA receptor desensitization, cyclothiazide (CTZ). The solvent dimethyl sulfoxide (DMSO) is commonly used to dissolve this drug, as well as many others. Unexpectedly, the vehicle alone already at 0.02%, but not at 0.01%, caused significant increases in EPSP amplitudes and NMDA-induced locomotor frequency. The results indicate that DMSO may have a profound influence when used at or above 0.02%, a concentration 10-50 times less than those most commonly used. Subsequently we applied CTZ at or below 10 µM concentrations (DMSO ≤ 0.01%). CTZ (1.25-5 µM) caused a marked and significant increase in EPSPs mediated by non-NMDA receptors and in both AMPA- and NMDA-induced locomotor frequency, but no effects on EPSPs mediated by NMDA receptors. From the effects of CTZ it is apparent that AMPA receptor desensitization plays an important role in determining locomotor frequency and that this is likely due to its limiting function on AMPA receptor mediated EPSPs.
INTRODUCTION

The neuronal network coordinating vertebrate locomotion is located in the spinal cord, and the cumulative evidence indicates that the network is normally activated from the brainstem via activation of N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and kainate receptors, as studied in the lamprey and other species (Brodin and Grillner 1985; Brodin et al. 1985; Deliagina et al. 2000; El Manira et al. 1997; Grillner et al. 1981; McClellan and Grillner 1984; Ohta and Grillner 1989; Sirota et al. 2000; for reviews see Grillner et al. 2000; Grillner and Wallén 1999). Under experimental conditions the lamprey network can also be activated by superfusing the isolated spinal cord with glutamate receptor agonists to elicit fictive locomotion (Alford and Grillner 1990; Brodin et al. 1985; Cohen and Wallén 1980; Grillner et al. 1981). Many studies have focused on NMDA receptors, particularly with respect to their voltage-dependent properties. Few studies, however, have been specifically aimed at the role of AMPA receptors in the network, and none on the possible importance of their desensitization in shaping the rhythmic activity during locomotion.

AMPA receptors in general are characterized by their rapid desensitization, high single-channel conductance and low affinity (Mayer and Armstrong 2004; Patneau et al. 1993; Tang et al. 1989; Trussell and Fischbach 1989). The role of desensitization is of great interest, because it may modulate the shape of synaptic transmission, regulate the activity of postsynaptic receptors and protect neurons from the neurotoxic effect of glutamate (Trussell and Fischbach 1989; Zorumski and Thio 1992). During the last few years several studies have reported the effects of desensitization on signal transmission (Funk et al. 1995), AMPA-receptor channel kinetics (Yamada and Rothman 1992) and individual EPSCs (Vylicky et al. 1991), while the action of desensitization of AMPA and NMDA receptors mediating EPSPs in locomotor networks has remained largely uninvestigated.
Selective modulators of glutamate receptor desensitization, such as concanavalin A, aniracetam, 4-[2-(phenylsulfonylamino)ethylthio]-2,6-difluoro-phenoxyacetamide and cyclothiazide (CTZ) have been described in recent years (Isaacson and Nicoll 1991; Johansen et al. 1995; Wong and Mayer 1993; Yamada and Tang 1993), which gave us the possibility to reinvestigate the relationship of AMPA receptor properties to the spinal network for locomotion. CTZ is the most potent and well known of the benzothiadiazides and it selectively and effectively reversibly blocks AMPA-receptor desensitization (Mayer and Armstrong 2004; Patneau et al. 1993; Yamada and Rothman 1992), the molecular mechanism of which has recently been detailed (Leever et al. 2003; Sun et al. 2002).

CTZ has been shown to increase rise and decay of mEPSCs in rat cortex (Atassi and Glavinovic 1999; Hestrin 1992), and to significantly enhance respiratory network motor output and synaptic drive currents to respiratory XII motoneurons (Funk et al. 1995). To gain an understanding of the role of AMPA receptor desensitization with regard to the locomotor network, we conducted an analysis of the effects of CTZ in the lamprey spinal cord.

CTZ is water-insoluble and is usually dissolved in dimethyl sulfoxide (DMSO), which is widely used as a solvent in neuroscience research. However, vehicle controls have not always been reported and the possible effects of DMSO on synaptic and/or network interactions have often not been thoroughly examined. DMSO has, however, been shown to have effects on its own (Birder et al. 1997; Kubota et al. 1998; Lu and Mattson 2001; Maclennan et al. 1996; Nakahiro et al. 1992), and we therefore examined the effects of low concentrations of DMSO (0.005-0.02%) alone, on AMPA- and NMDA-induced locomotion, and on excitatory AMPA and NMDA receptor EPSPs. It turned out that DMSO in itself has effects (at 0.02% or higher) at concentrations 10-50 times less than commonly used. Our results should be taken into account for all experiments that involve glutamatergic neurotransmission in which DMSO is used as a solvent for various drugs.
This study shows that CTZ, at low concentrations, affects AMPA receptor mediated EPSPs and AMPA- and NMDA-induced fictive locomotion in lamprey. The results have been presented in abstract form (Tsvyetlynska et al. 2004).

FIG. 1. Preparation arrangement and the effects of glutamate receptor antagonists. A: Illustration of reticulospinal (rs) axon stimulation and intracellular (i.c.) recording on a piece of spinal cord. B: Blocking NMDA receptors with AP5 reduced EPSPs evoked by stimulating ventromedial axons extracellularly. C: Similar reductions were observed when the non-NMDA receptors were blocked by CNQX. All traces are averages of 50 responses.

METHODS

Animals and dissection

Adult lampreys (Lampetra fluviatilis, n = 62) were obtained from Sweden or Finland, kept in an aquaria, cared for, and sacrificed according to an accredited protocol and guidelines outlined by
the North Stockholm Ethical Committee. The animals were anaesthetized with tricaine methane sulfonate (100 mg/l) and decapitated caudal to the gills. Pieces of isolated spinal cord 10-20 segments in length, with meninges previously removed, were pinned down with the ventral side up in a cooled chamber. The chamber was lined with a silicon elastomer (Sylgard) and the temperature of the continuously perfused Ringer’s solution was maintained at 4-8 °C. The physiological solution was composed of (in mM) 138 NaCl, 2.1 KCl, 1.8 CaCl2, 1.2 MgCl2, 4 glucose, 2 HEPES and 0.5 L-glutamine. It was bubbled with O2 for 20 min and then adjusted to pH 7.4 using 1 M NaOH.

**Chemicals**

Fictive locomotion was induced by adding 50 µM NMDA (Tocris, Bristol, UK) or 1 µM AMPA (Tocris, UK) to the perfusate. In some experiments, in order to isolate the non-NMDA and NMDA components, we applied the NMDA receptor antagonist D(-)-2-amino-5-phosphonopentanoic acid (AP5, 50 & 100 µM) and, correspondingly, the AMPA receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 40 µM) to the bath (Alford and Grillner 1990). Drugs were made as 1000× stock solutions in aliquots for single use and kept frozen at -20°C; the solutions were diluted to the final concentration of drugs before each experiment. Muscimol hydrobromide (Sigma, St. Louis, USA) was diluted to 50 µM from frozen 100 mM stock solutions in Ringer’s.

Cyclothiazide (CTZ, Tocris, Bristol, UK), a potent blocker of AMPA receptor desensitization, was dissolved in DMSO (100 mM stock solution) and was then diluted to 1.25-10 µM for bath application (Arai and Lynch 1998; Patneau et al. 1993). In order to investigate the effect of CTZ on the AMPA- and NMDA-induced locomotion and AMPA- and NMDA-mediated EPSPs, we performed a cumulative dose response study. The concentration of CTZ was
increased progressively every 10 min alternated with washout periods (10 min). In order to investigate the effects of DMSO alone, a concentration-response study was performed at concentrations of 0.001%-0.02% (corresponding to CTZ concentrations up to 20 µM).

Electrophysiology

To determine the effects of CTZ and DMSO on fictive locomotion, we performed ventral root recordings with glass extracellular suction electrodes. The signals were then amplified with a differential AC Amplifier (model 1700, A-M Systems, Carlsborg, WA, USA) and band-pass filtered at 300 Hz – 1 kHz. EPSPs from neurons were obtained using sharp electrodes from borosilicate or aluminosilicate glass filled with 3 M KCl (30–60 MΩ resistance) and amplified with an Axoclamp 2A (Axon Instruments) coupled to a DC amplifier to further increase the gain, which was necessary for good digital resolution of the A to D converter.

Intracellular recordings were made from the somata of grey matter neurons while stimulating presynaptic axons, of reticulospinal origin, with an extracellular suction electrode placed superficially on the ventromedial surface of the spinal cord (Ohta and Grillner 1989) (Fig. 1A). EPSPs with a monosynaptic component were identified by their ability to follow a 2 Hz presynaptic stimulus artifact reliably and with constant latency. Stimulus pulses (1 ms, 1-2 mA) were delivered to the medial, ventral surface of the spinal cord via glass suction electrodes using an isolated stimulator (Model 2100, A-M Systems, Inc., Carlsborg, WA). As the stimulus electrode placement was one cm or more from the recording electrode, and because the only large diameter, axons with a low stimulus threshold in the ventromedial area near the ventral surface that run the length of the spinal cord are reticulospinal axons, the shorter, smaller axons lying deeper dorsally, the major component of the responses was due their activation.
Data analyses were performed using pClamp software version 8.2 (Axon Instruments). Stimulus-evoked EPSPs were averaged for 20-50 consecutive sweeps at the various recording times, and the amplitudes were measured from baseline to the first peak. Traces of fictive locomotion were analyzed to determine the average burst frequency by using 20 consecutive alternating bursts. Further analysis was performed with Excel software (Microsoft). The effects of CTZ or DMSO were compared to controls using Student's $t$-test. The accepted confidence level for significance was always 95%. Data are expressed as means ± standard error of the mean (S.E.M.).

RESULTS

EPSPs evoked by the stimulus protocol are mediated by both NMDA and non-NMDA receptors

It was important to separate the NMDA- and non-NMDA-mediated components of EPSPs in the lamprey spinal cord using our stimulus protocol. This was accomplished by blocking the NMDA receptors with AP5 (50 µM) (Fig. 1B) and non-NMDA receptors with CNQX (40 µM) (Fig. 1C) while recording EPSPs in neurons elicited by stimulation of reticulospinal axons.

Low concentrations of DMSO can increase non-NMDA- and NMDA-mediated EPSPs

We first investigated the effects of DMSO alone in order to determine any side effects of this vehicle and, if there were such, to assess the concentration necessary to avoid them. The effect of DMSO in concentrations from 0.01% to 0.02% on both non-NMDA- and NMDA-mediated EPSPs was examined. The EPSP amplitudes were averaged from 50 EPSPs for each condition for each cell.
FIG. 2. Effect of different concentrations (0.01%-0.02%) of DMSO on reticulospinal non-NMDA and NMDA receptor mediated EPSPs. A, B: Postsynaptic responses of two spinal neurons to reticulospinal axon stimulation, before, during and after wash out of DMSO applications, where in one the NMDA component was blocked by bath application of 50 µM AP (A), and in the other the non-NMDA component was blocked by 40 µM CNQX (B). In both neurons, 0.01% DMSO had little effect on EPSP amplitude whereas 0.02% caused a marked increase. Each trace is an average of 50 EPSPs where the baselines ranged –79 to –84 mV. C, D: Cumulative dose-response relationships from individual cells for each condition, showing changes of the EPSPs amplitude during application of different concentrations of DMSO. Both graphs show that 0.01% DMSO had no effect in three of four neurons on the amplitude in AP5 and on two of three neurons in CNQX. In 0.02% DMSO there were significant increases in the amplitude in three of three and three of four cells in AP5 and CNQX respectively. * = P < 0.05, ** = P < 0.01, *** = P < 0.005, ns = not significant, s.a. = stimulus artifact.
Figure 2A shows averaged EPSPs from a neuron in the presence of AP5 in control and at DMSO concentrations of 0.01% and 0.02% DMSO. At 0.01% DMSO had little effect whereas 0.02% clearly increased the size of the EPSPs. The results from the individual experiments with AP5 are graphically illustrated in Figure 2C, where there were no significant increases in EPSP amplitude with 0.01% DMSO (n = 4), but in the three experiments where DMSO was increased to 0.02% the EPSPs were significantly larger in all cases. DMSO caused no significant increases in the amplitude of NMDA receptor mediated EPSPs at concentrations of 0.01% (n = 3) (Fig. 2B,D). When the concentration was raised to 0.02% DMSO, the amplitude was significantly increased in three of four neurons (Fig. 2D). These findings suggest that DMSO concentrations at 0.01% have little influence on either non-NMDA- or NMDA-mediated EPSPs, whereas a concentration of 0.02% potentiates both non-NMDA- and NMDA-mediated glutamatergic synaptic transmission.

**DMSO above 0.01% influences NMDA – and AMPA-induced fictive locomotion**

Fictive locomotor activity was induced by superfusing the spinal cord with a Ringer’s solution containing 50 µM NMDA or 1 µM AMPA while recording the locomotor activity from the ventral roots. We first tested the effect of DMSO on NMDA-induced fictive locomotion at very low (0.005%-0.02%) concentrations. Figure 3A shows the locomotor patterns recorded under control conditions and with the addition of DMSO in increasing concentrations separated by NMDA washes. The pharmacological effect was similar to previous data in that there was a marked increase in frequency by 50 ± 14% with the addition of 0.02% DMSO (Fig 3B). In all three experiments the effect of DMSO was not significant in concentrations at or below 0.01%.
FIG. 3. NMDA-evoked fictive locomotion during gradually increasing DMSO concentrations. A: Extracellular ventral root recordings with continuous perfusion in 50 µM NMDA under control conditions (1.56 ± 0.01 Hz), in the presence of DMSO (concentrations ranging from 0.005% to 0.02%) and after washouts. An increase in frequency followed only after application of 0.02% DMSO. B: Burst frequencies from three experiments showing, no significant changes for concentrations at 0.01% or less, but significant increases in all three experiments with 0.02% DMSO. Burst frequencies are expressed as a percent of the averaged response values collected during the 10-min period preceding the infusion of the DMSO. ** = $P < 0.01$, *** = $P < 0.005$, ns = not significant.
FIG. 4. The modulatory effects of cumulatively increased CTZ and DMSO concentrations on AMPA-induced fictive locomotion. Extracellular recordings from ventral roots in AMPA (1µM) alone, showing irregular initial locomotor rhythm at high amplification. Application of a low dose of CTZ (1.25 µM) led to stable bursting. CTZ was applied sequentially from low to high concentrations (1.25-10 µM), and recordings were made following 10 minutes equilibration at each dose. These were separated by 10-minute periods of application of DMSO alone at the concentration equivalent to the next application of CTZ. Increasing the dose of DMSO alone did not result in a further increase in frequency beyond that seen with the preceding application of CTZ. However, with each increase in CTZ concentration the burst frequency increased markedly, above that seen with the preceding dose of CTZ.
We next investigated the effect of DMSO on AMPA-induced locomotion. Because the application of AMPA (1 µM) tended to produce less regular locomotion, we added CTZ in order to achieve a stable rhythm as well as to examine the role of AMPA receptor desensitization (see below). Figure 4 illustrates fictive locomotion recorded in an experiment in the presence of 1µM AMPA and incremented increases in DMSO concentration from 0.005% to 0.01% during the 10-minute intervals between doses of CTZ. No significant differences in frequency were observed with DMSO concentrations at or less than 0.01%. Because of these results and those with EPSPs we therefore performed the following experiments with CTZ using concentrations of DMSO below or at 0.01%.

*Cyclothiazide modulates the frequency of AMPA-induced fictive locomotion*

Fictive swimming was induced in isolated spinal cord preparations by perfusion of 1 µM AMPA. CTZ was applied to the bath by cumulatively increasing the dose. The concentration of CTZ was increased progressively from 1.25 µM to 2.5, 5.0 and 10 µM at 10 min intervals. Figure 4 shows a representative example of the dose-dependent increases in the frequency of fictive locomotion beginning with an increase in concentration from 1.25 to 2.5 µM (3.4 Hz as compared to 2.2 Hz). As illustrated, with AMPA alone the rhythm was somewhat irregular. We therefore used 1.25 µM CTZ as the baseline data for comparison. When 10 µM CTZ was applied, the burst frequency increased markedly, to above 5 Hz (Fig. 4). The three individual experiments are graphically illustrated in Figure 5A, which shows significant increases in frequency with each elevation in CTZ concentration above 1.25 µM. Figure 5B shows the combined, normalized means of the experiments. The effect on frequency was thus dose-dependent from 1.25 µM to 10 µM. At the highest concentration the mean increase in burst frequency reached 252 ± 48% of the 1.25 mM CTZ controls (Fig. 5B; n = 3).
To investigate the influence of CTZ at the synaptic level, non-NMDA-mediated EPSPs evoked by reticulospinal axon stimulation were also investigated. As illustrated in Figure 6, A-C, bath application of CTZ (1.25-5.0 µM) in the presence of AP 5 (50 µM) increased the amplitude of the EPSPs already at a concentration of 1.25 µM in spinal neurons during stimulation of reticulospinal axons (n = 3, P < 0.001). The maximum amplitude was reached at a concentration of 10 µM CTZ (data not shown). The enhancement recovered by at least 50 % after 10 minutes of washout (Fig. 6A-C). Figure 6D shows the increase in the average EPSP amplitude in the presence of 50 µM AP5 in controls and after application of three different concentrations of CTZ in six individual experiments. Data were normalized from six experiments (Fig. 6E) to present a dose-response of EPSPs to CTZ in different neurons, which were significantly (P < 0.05)
enhanced by 1.25 and 2.5 µM, and 5.0 µM CTZ compared to controls. These results suggest that AMPA receptor desensitization has functionally significant effects on synaptic responses within the neuronal network and that this may account for the increase in frequency observed during fictive locomotion.

*Cyclothiazide increases the frequency of NMDA-induced fictive locomotion, during endogenous activation of AMPA receptors*

To investigate if a gradual increase in CTZ concentration could influence NMDA-induced fictive locomotion, as seen with AMPA-induced locomotor activity, similar concentrations (5.0 µM, 10 µM) were slowly applied to the recording chamber during NMDA-induced (50 µM) fictive locomotion. Any effects observed here would most likely be due to modulation of AMPA receptors during activation mediated by an endogenous release of glutamate. Figure 7A shows typical rhythmic ventral root bursts induced by NMDA and those with the addition of CTZ. As shown, both 5.0 and 10 µM CTZ resulted in an increase in the burst frequency. Individual concentration-response records from four different experiments are illustrated in Figure 7B, showing that CTZ caused a significant increase in burst frequency at a concentration of 5.0 µM in three of four experiments. With further CTZ application (10 µM), we did not observe a significant difference in burst frequency from that at 5.0 µM in three of four experiments. The combined results (Fig. 7C) show that 5.0 µM CTZ caused a significant increase in the ventral root burst frequency to 152 ± 27% of controls after 10 ± 4 min application. Thus, CTZ also increased the frequency of NMDA-induced locomotion, presumably due to block of desensitization of AMPA receptors activated by network interneurons that would lead, in turn, to an increased excitatory drive. To control for possible effects of CTZ on GABA_A receptors, we
FIG. 6. CTZ enhances the amplitude of non-NMDA- but not NMDA-mediated EPSPs. A: intracellular recordings from a spinal neuron, showing non-NMDA receptor-mediated EPSPs under basal conditions (in the presence of 50 µM AP 5), after 10 min of perfusion with a solution containing 1.25 µM CTZ, and 10 min after wash out of CTZ. B, C: Similar records with solutions containing 2.5 µM and 5.0 µM CTZ respectively, showing further increases in amplitude with each dose. D: Average EPSP amplitudes (N = 50 traces) plotted during application of CTZ (1.25 – 5.0 µM) in six different experiments. E: Summary of six experiments showing, for concentrations from 1.25 to 5.0 µM, significant increases (P < 0.05 for each concentration) in EPSP amplitude compared to control values. S.E.Ms are presented as T-bars. * = P < 0.05, ** = P < 0.01, ***
FIG. 7. Effect of CTZ on NMDA-induced fictive locomotion. A: ventral root recordings of the rhythmic activity induced by NMDA (50µM). The rhythm was increased by application of 5.0 µM CTZ, fell to control levels after 10 min wash out, and then again increased at 10 µM CTZ without significant additional effects. B: Representative plot with changes of burst frequency in four different animals exposed to increasing concentrations of CTZ. Each point is an average of the frequency of 20 consecutive bursts. C: Graph summarizing the effects of CTZ at concentrations 5.0-10 µM on burst frequency. Data from four experiments are shown. D: Illustration of lack of effect of 5 and 10 µM CTZ on NMDA EPSPs. E: Mean NMDA EPSPs during control and CTZ application shown in graphical form for three experiments. Vertical T bars = S.E.M, * = P < 0.05, ** = P < 0.01, *** = P < 0.005, ns = not significant.
briefly applied pulses of 50 µM muscimol over a spinal neuron during intracellular recording. The amplitudes of the hyperpolarizing responses were not different before and after application of 10 µM CTZ. NMDA receptor-mediated actions are known not to be affected by CTZ (Ballerini et al. 1995; Dougherty et al. 1998; Hoyt et al. 1995; Lin et al. 2002; Yamada and Tang 1993), and we confirmed this in three experiments. Thus, application of 10 µM CTZ in the presence of 40 µM CNQX, where EPSPs were mediated by NMDA receptors, did not significantly change the mean EPSP amplitude in three experiments (Fig. 7B,C).

DISCUSSION

Effect of DMSO on reticulospinal AMPA- and NMDA-mediated EPSPs and fictive locomotion

DMSO has been widely used in neurophysiological studies to increase the solubility of water-insoluble drugs, including CTZ, at concentrations from 0.05 to 10 % with 0.5-1% often being used (Lu and Mattson 2001). We report here that even at a very low concentration (0.02%), which is 10-50 times less than that commonly applied, DMSO significantly increases the amplitude of both NMDA-and non-NMDA-mediated EPSPs and caused a significantly higher frequency of NMDA-induced locomotion. This suggests that DMSO at or above this concentration may affect glutamatergic synaptic transmission and fictive locomotion. DMSO had no significant effects on non-NMDA receptor-mediated EPSPs in three of four neurons or on fictive locomotion in lamprey at concentrations at or less than 0.01%, actually corresponding to CTZ-concentrations up to 10 µM. To guard against effects of DMSO, in our experiments the concentration of CTZ was never more than 10 µM.

At cholinergic synapses, many investigations have attempted to elucidate the mechanism underlying the action of DMSO (Gandiha and Marshall 1972; Geron and Meiri 1985;
Kubota et al. 1998; Matsumoto et al. 1985; Sawada and Sato 1975; Stolc and Vlckova 1982), while the action of this solvent on glutamatergic synaptic transmission and fictive locomotion has not been investigated. In the case of enhanced cholinergic EPSPs and IPSPs, several mechanisms were suggested including inhibition of cholinesterase activity and increased presynaptic Ca\(^{2+}\) entry at low concentrations of DMSO. Others have reported a decrease in postsynaptic responses and postulate a direct effect on glutamate receptors (Lu and Mattson 2001). In those studies the concentration of DMSO was high, up to several hundred times the highest used in our experiments. The actual mechanism responsible for the effects in glutamatergic synaptic transmission is beyond the scope of this study. However, reports by others, cited below, suggest several possibilities that could explain our results.

DMSO has been shown to enhance the Ca\(^{2+}\) influx into presynaptic nerve terminals (Matsumoto et al. 1985) and to increase the frequency of miniature endplate potentials (Geron and Meiri 1985). DMSO could act presynaptically by increasing transmitter release due to enhanced Ca\(^{2+}\) entry, and thus raise the level of excitability of the locomotor network. Because both NMDA and AMPA responses were enhanced to roughly the same degree in our study, and since it seems unlikely that the same postsynaptic effects would occur on receptors composed of different classes of protein subunits, we favor the hypothesis of a presynaptic action common to glutamate release. This is in line with the earlier evidence of a DMSO-induced increase in presynaptic Ca\(^{2+}\) influx. This, along with the fact that our DMSO concentrations were below the level of synaptic and network effects, and that CTZ is now recognized as having a highly specific conformational effect on glutamate receptor proteins (Leever et al. 2003; Nakagawa et al. 2005; Sun et al. 2002), no link between the effects of CTZ and DMSO are supported. The actions reported here on the effect of low concentrations of DMSO (0.005- 0.02%) on glutamatergic synaptic transmission and fictive locomotion should be considered in future neurophysiological studies in which DMSO is used as a solvent for various pharmacological agents.
Effects of CTZ on the frequency of AMPA-induced fictive locomotion and AMPA-mediated EPSPs

We report here the degree of CTZ-induced modulation of fictive locomotion at low and high concentrations in the presence of 1 µM AMPA. The frequency of fictive locomotion was significantly increased already at a concentration change from 1.25 to 2.5 µM CTZ. As fictive locomotion was induced by bath-applied AMPA, the modulation by CTZ may have been partially mediated by a depressed desensitization of AMPA receptors activated exogenously. However, as a clear enhancement of EPSPs was observed (see below), the effects of CTZ on synaptic transmission undoubtedly play a major role in the change in burst frequency. Further, CTZ has been reported to have similar effects in other systems where the activity was not induced by AMPA application. CTZ modulated network behavior, increased respiratory network burst frequency, peak, and integrated amplitudes of network motor output in hypoglossal respiratory motoneurons (Funk et al. 1995). After application of CTZ all lumbar motoneurons of the rat isolated spinal cord displayed an increase in the frequency of depolarizing spontaneous events and subsequent bursting activity (Ballerini et al. 1995). Our present study demonstrates that the most plausible explanation for the elevation in frequency of the locomotor rhythm is that CTZ potentiates the AMPA receptor-mediated synaptic interactions underlying generation of fictive locomotion, and that the desensitization process of AMPA receptors undoubtedly substantially contributes to network properties.

Bath application of CTZ in the presence of the NMDA receptor antagonist AP5 increased the amplitude of the reticulospinal EPSPs by approximately 100-400%. In previous studies of brainstem and hippocampal slices, CTZ prolonged the decay of the fast EPSC mediated by AMPA receptors (Barnes-Davies and Forsythe 1995; Rammes et al. 1994), and increased the
amplitude of EPSCs (Arai and Lynch 1998; Ishikawa and Takahashi 2001). The increase in amplitude seen in the present study after CTZ exposure presumably results from a similar prolongation of the underlying EPSC.

There are two possible actions of CTZ on receptor channel gating that could affect the shape of charge transfer for individual EPSPs, which in turn would alter synaptic transmission and network activity: block of rapid desensitization, or delayed receptor deactivation. There is strong evidence that CTZ stabilizes the AMPA receptor configuration after ligand binding, thus preventing collapse to the desensitized state (Sun et al. 2002). The observed effects of CTZ on lamprey neuron EPSPs, then, are likely due to occlusion of desensitization and not to delayed deactivation.

As in the present study, CTZ significantly increased the amplitude of synaptic responses mediated by glutamate receptors in the calix of Held (Ishikawa and Takahashi 2001) and the endbulb of Held (Bellingham and Walmsley 1999). Both studies concluded that the effects were primarily presynaptic in origin. However, as discussed above, DMSO at concentrations used in those studies (0.1-0.3%) may have caused enhancement of presynaptic transmitter release, whereas in our experiments DMSO concentrations were well below those shown to have effects from the solvent alone (0.00125-0.01%). Moreover, it is unlikely that a presynaptic effect in this case could account for the findings since AMPA EPSPs were affected by CTZ but not NMDA-mediated EPSPs in the same preparations.

Effects of CTZ on the frequency of NMDA-induced fictive locomotion

We also observed that CTZ caused a significant increase in the frequency of NMDA-induced fictive locomotion. As CTZ had no significant effects on NMDA receptors in the present study and in those reported by others (Ballerini et al. 1995; Dougherty et al. 1998; Hoyt et al. 1995; Lin et al. 2002; Yamada and Tang 1993), the observed increase in burst frequency was most likely
due to effects on AMPA receptors. This finding indicates that AMPA receptor desensitization, be it modulated or constant, is important during endogenous glutamate release. CTZ has been reported to inhibit GABA<sub>A</sub> receptors (Deng and Chen 2003), and block of these receptors can increase the frequency of NMDA-induced fictive locomotion (Schmitt et al. 2004). In control experiments the highest concentration of CTZ used (10µM) had no effect on GABA<sub>A</sub> receptors. But also, as the increases in EPSPs in the present study were significantly enhanced at concentrations (1.25-10 µM) near the threshold of those shown to block GABA<sub>A</sub> receptors, which had an IC<sub>50</sub> of 57 µM (Deng and Chen 2003), our observed frequency increase at the concentrations used is unlikely due to inhibition of these receptors.

From our results and those cited above we can conclude that the increase in frequency of NMDA-induced fictive locomotion in the presence of CTZ is due to prolonged AMPA receptor activation that in turn leads to an increase in the overall glutamatergic drive of locomotor activity. As CTZ had no effects on NMDA receptors, gap junctions (see Fig. 2A) or GABA<sub>A</sub> receptors, but did have a profound effect on non-NMDA receptors, it most likely exerts its effects on the network via the latter type of receptors. In view of this and the cited evidence of the specificity of CTZ for AMPA receptors, we thus suggest that desensitization of AMPA receptors has a marked influence on glutamatergic synaptic transmission and plays a significant role in shaping the network properties of the spinal cord. The action of CTZ in reducing AMPA desensitization is thus similar in lampreys and mammals, which means that the basic molecular structure underlying desensitization was presumably present already 450 million years ago when cyclostomes diverged from the main vertebrate line.

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