Ethanol and magnesium suppress nickel-induced bursting activity in leech Retzius nerve cells

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Abstract. In the present study we have examined effects of ethanol and magnesium on Ni^{2+}-induced bursting of leech Retzius cells. Saline with 3 mmol/l $NiCl_2$ induced spontaneous bursting activity, characterized by rapid depolarizations to a plateau level during which bursts of action potentials occurred. To test for the mechanism of bursting initiation external Na^+ was completely removed. Removal of external Na^+ in presence of 3 mmol/l $NiCl_2$ terminated the bursting activity. Application of 2% ethanol solution significantly decreased the bursting frequency, duration and amplitude of depolarization plateaus, and the number of spikes per plateau. Solution containing 10 mmol/l Mg^{2+} almost completely abolished the oscillatory activity of the neurons and completely suppressed action potential generation. We conclude that ethanol and magnesium suppress Ni^{2+}-induced epileptic activity.

Key words: Bursting — Leech Retzius neuron — Ethanol magnesium

Introduction

Epileptic seizures are based on paroxysmal depolarization shifts (PDS) which are synchronized in many neurons. Mechanisms underlying PDS and seizures are still not understood (Üre and Altrup 2006). Essential mechanisms of epileptic activity are thought to be identical in whatever part of the human or animal nervous system it appears (Altrup 2004). Invertebrate models have proved to be quite useful for the understanding of some processes in the central nervous system. Since invertebrate neurons are easily accessible to experimentation, it has been possible to explore in detail the basic mechanisms controlling neuronal excitability using these cells and to make some useful predictions about electrophysiological mechanisms that may be present in the central neurons (Lewis et al. 1986).

The nervous system of the leech is a valuable model system for investigating the cellular basis for epileptiform activity. As in other invertebrate preparations, it has been possible to identify many of the individual neurons comprising the relevant neuronal circuits and to determine their cellular and synaptic properties (Angstadt 1999). The largest neurons in the leech central nervous system are Retzius cells which exhibit stable resting membrane potential and which are nonbursting neurons with a low spontaneous firing rate (Beck et al. 2001).

Rhythmical epileptiform bursting activity can be induced in the Retzius neurons by several exogenous agents, such as neutral red, various pyrethroids (Leake 1982) and by the convulsants, penicillin or bemegride (Prichard 1972). Furthermore, epileptiform activity can be induced by FMRF-amide or related peptides, by the injection of phorbol ester, and by lowering external Ca^{2+} concentration through reducing $CaCl_2$ in bath media with or without EGTA, the use of EGTA alone, and by replacement of external Ca^{2+} by Ba^{2+}, Co^{2+}, and other inorganic Ca^{2+}-channel blockers, such as Ni^{2+}, Mn^{2+}, Cd^{2+}, La^{3+} and Zn^{2+} (Angstadt et al. 1998). This epileptiform activity has been observed not only in Retzius neurons, but in nearly all neurons in leech ganglia, perhaps synchronized by electrical coupling. In Na^+-free Ringer solution epileptiform activity induced by Ni^{2+} becomes eliminated, suggesting that sodium influx plays a major role in its generation (Angstadt and Friesen 1991).

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Anticonvulsant effects of ethanol have been revealed by a number of *in vivo* studies in various electrically- and chemically-induced seizures tests (Zuk et al. 2001; Hillbom et al. 2003). Similarly, magnesium has an anticonvulsant effect in preeclampsia and eclampsia (Sibai 1990), and magnesium levels are shown to be significantly lower in patients with seizures (Sinert et al. 2007). *In vitro* and *in vivo* experiments have also provided evidence for the ability of magnesium to affect seizures, showing the anticonvulsant effect of magnesium (Standley et al. 1995; Wang et al. 2004).

Since ethanol and magnesium both show inhibitory effect on epileptiform activity, by influencing ion channel function and synaptic transmission, we have undertaken a study to test whether they could modulate this Ni^{2+}-induced Na^{+}-dependent bursting activity, which greatly resembles the electrophysiological properties of neurons during seizures.

Materials and Methods

The experiments were performed at room temperature (22–25°C) on Retzius nerve cells in the isolated segmental ganglia of the ventral nerve cord of leech *Haemopis sanguisuga*. The method of dissection has been previously described (Beleslin 1971), and complies with institutional research council guidelines.

Dissected segments of 3 ganglia were immediately transferred to 2.5 ml plastic chamber with leech Ringer and fixed by means of fine steel clips. The plastic chamber was then placed in grounded Faraday's cage mounted on a fixed table in a manner that prevents vibrations. Identification and penetration of the cells was performed in the cage under a stereomicroscope. The Retzius cells were identified by their position on the ventral surface of the ganglion, their size, and their bioelectrical properties.

Prior to the experiments, the chamber was flushed with fresh Ringer solution, microelectrode dipped into the solution and allowed 20–30 min for equilibration. To change the solution, the chamber was continuously flushed with a volume of fluid at least 5 times that of the chamber volume. The superfusion was usually completed in 10–15 s.

Electrical methods (electrophysiological recordings)

The membrane potential was recorded using standard single-barrel glass microelectrodes. Micropipettes were pulled from thick wall capillaries with internal filament (O.D. 1.5 mm, I.D. 0.6 mm, World Precision Instruments) on a vertical puller (Narishige, Japan) and then filled with 3 mmol/l KCl shortly after being pulled. The tip diameter of the electrodes was less than 1 μm, tip potentials were less than 5 mV, and the microelectrode resistance was 15–25 MΩ in standard Ringer solution (for composition see solutions).

The recordings were amplified using high input impedance amplifier (model 1090, Winston Electronics). Microelectrodes were connected to the amplifier *via* an Ag-AgCl wire. The ground electrode was an Ag-AgCl wire in a separate chamber filled with Ringer solution connected to the experimental chamber by a 3 mmol/l KCl 3% agar bridge. The recordings were displayed on a two-channel oscilloscope (Hameg, Germany) and permanently recorded on a pen recorder (Linseis, Selb, Germany), and a thermal printer (Hameg, Germany).

Solutions

The Ringer solution used in these experiments had the following composition (in mmol/l): NaCl 115.5, KCl 4, $CaCl_2$ 2, NaH_2PO_4 0.3, Na_2HPO_4 1.2 (pH = 7.2). In Ni^{2+}-containing solutions 3 mmol/l $NiCl_2$ was added. In Na^{+}-free solutions NaCl was completely replaced by an equal amount of tris(hydroxymethyl)aminomethane-Cl (Tris), Na_2HPO_4 and NaH_2PO_4 were omitted, and pH adjusted to 7.2 with HCl. Absolute ethanol (Merck) was added to the Ringer solution to produce a final concentration of 2% ethanol. In magnesium containing saline 10 mmol/l $MgCl_2$ was added and NaCl was reduced by 15 mmol/l.

Data analysis

All results are expressed as means ± S.E.M. Comparison between mean values was made using Student's *t*-test; *p* values of less than 0.05 were considered significant.

Results

Ni^{2+}-induced bursting activity of Retzius neurons of H. sanguisuga

The resting membrane potential of the Retzius neurons in standard Ringer solution was –40.7 ± 1.6 mV (n = 18 cells), and spontaneous action potentials of amplitude, shape and duration usual for leech Retzius cells (amplitude 20–50 mV, duration 6–8 ms) were generated at a low frequency (≈1 Hz, Fig. 1A). Superfusion with Ringer solution containing 3 mmol/l of Ca^{2+} channel blocker Ni^{2+} (Ni-Ringer) induced a small depolarization of the membrane potential followed by spontaneous bursting activity, characterized by rapid depolarizations to a plateau level during which bursts of action potentials occurred. The plateaus spontaneously ended by rapid repolarizations followed by interplateau intervals that over time gave way to the next rapid depolarization (Fig. 1B,C). Over the 2–7 min upon superfusion by Ni-Ringer the plateau depolarizations increased in frequency, duration, amplitude, and number of spikes per plateau, eventually reaching final

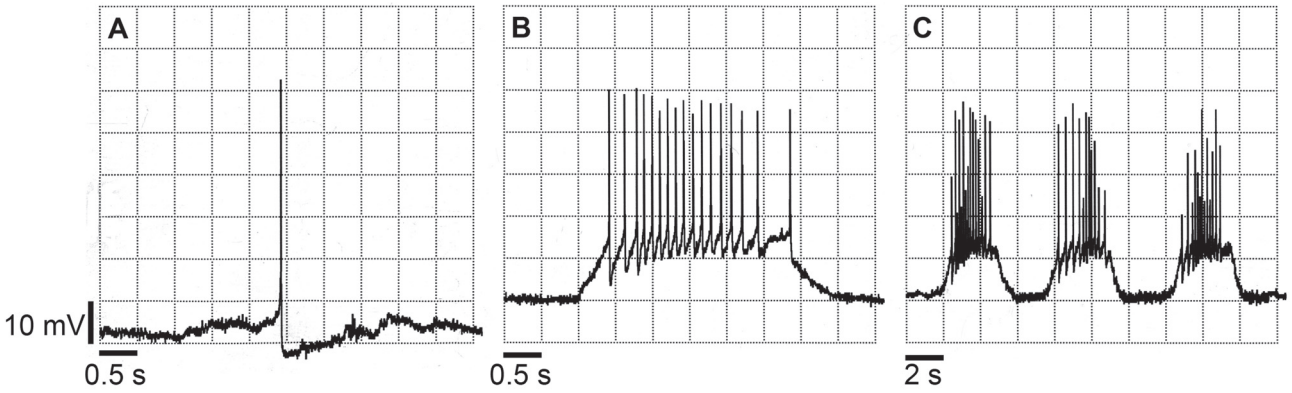

Figure 1. Spontaneous and bursting activity of leech Retzius neuron as recorded by thermal printer. **A.** Spontaneous firing of single action potentials in standard Ringer solution. **B.** A plateau of depolarization with burst of action potentials induced by 3 mmol/l $NiCl_2$. **C.** Same as B on a different time scale.

levels shown in Table 1. These levels were sustained for as long as Ni-Ringer was applied (Fig. 2). All further experiments were performed, and appropriate measurements taken, only when this final and stable level had been reached.

Bursting activity seized and the cells recovered within several minutes of washout with standard Ringer solution.

Dependence of Ni^{2+}-induced bursting activity on Na^+

Bursting activity induced in *Hirudo medicinalis* by Co^{2+}, Ni^{2+} (Angstadt and Friesen 1991), and other transitional metal ions (Angstadt et al. 1998) has been reported to be sodium-dependent (Angstadt and Friesen 1991). To examine whether the same holds for our preparation (*H. sanguisuga*), we have replaced Na^+ entirely by Tris in the Ni-Ringer solution (Tris-Ni-Ringer).

Bursting activity of 6.9 ± 0.7 plateaus per minute was induced by 3 mmol/l Ni^{2+} in Retzius neurons having a resting membrane potential of –39.7 ± 4.6 mV (n = 4 cells). Upon the stabilization of bursting activity Ni-Ringer was replaced by Tris-Ni-Ringer. Removal of external Na^+ in presence of 3 mmol/l Ni^{2+} induced a rapid hyperpolarization of the cell membrane. Within the first 2–3 min, the membrane potential partially depolarized and remained at a stable level for the remainder of Tris-Ni application. During the whole period of Tris-Ni application, the bursting activity was completely abolished since no plateaus of depolarization occurred. After washout with Ni-Ringer containing the normal concentration of Na^+, the cell membrane potential fully recovered and bursting activity resumed as before (Fig. 3).

Table 1. Characteristics of the Ni^{2+}-induced bursting activity of the Retzius neuron after stabilization

Parameter	Value	n
Spikes/plateau	7.09 ± 0.48	35
Plateau duration (s)	4.20 ± 0.16	45
Plateau amplitude (mV)	11.43 ± 0.54	47

Data shown as mean ± S.E.M.; n, number of plateaus used for measurements.

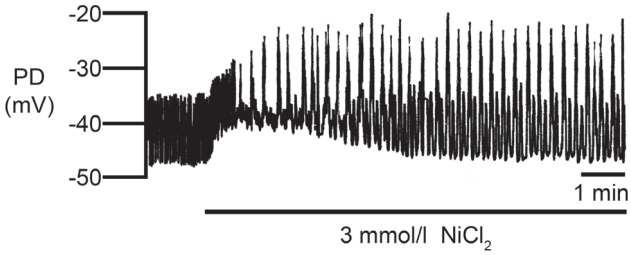

Figure 2. A trace from pen recorder showing bursting activity of leech Retzius neuron induced by 3 mmol/l $NiCl_2$. Application of Ni^{2+} is followed by initial depolarization and initiation of bursting activity. After 5 min membrane potential and bursts reach stable level that is sustained for the remainder of the recording. PD, membrane potential.

Effects of 2% ethanol on Ni^{2+}-induced bursting activity

In this set of experiments Ni-Ringer-induced bursting activity stabilized at 6.9 ± 0.7 plateaus per minute (n = 7 cells, resting membrane potential –41.0 ± 3.2 mV). Replacement of Ni-Ringer with 2% ethanol solution containing 3 mmol/l Ni^{2+} (EtOH-Ni-Ringer) led to a minor depolarization and a highly significant decrease in frequency of bursting activity to 2.9 ± 0.7 plateaus per minute ($p < 0.01$, Fig. 4A). Ethanol also adversely affected the duration and amplitude of the plateaus as well as the number of spikes per plateau (Fig. 4B). Plateau duration was reduced from 4.6 ± 0.5 s to 3.06 ± 0.36 s ($p < 0.05$, n =

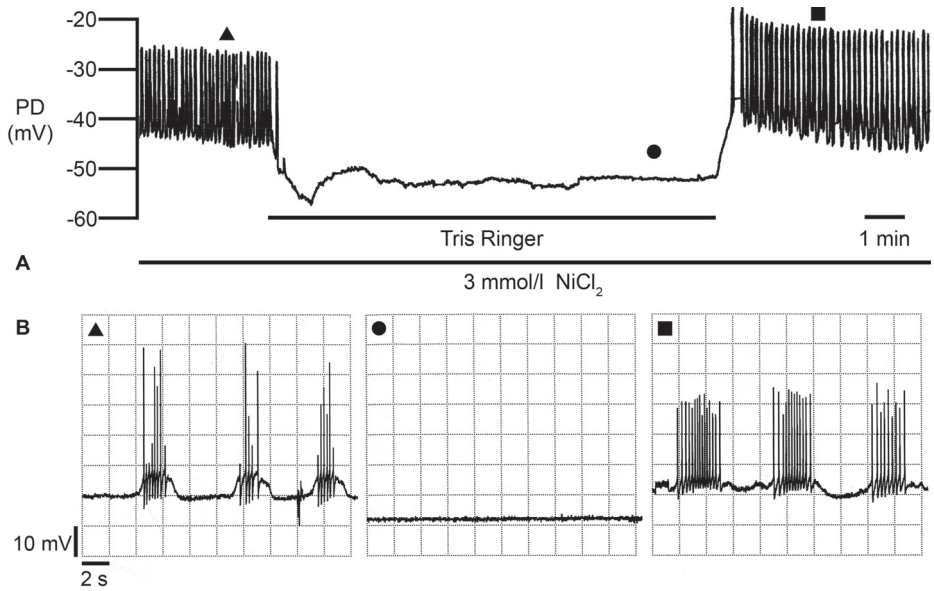

Figure 3. Effect of Tris Ringer on bursting of leech Retzius neuron. **A.** Trace from pen recorder. Upon application of Tris Ringer membrane potential transiently hyperpolarizes and bursting is completely terminated. Washout restores both membrane potential and bursting activity. Presence of $NiCl_2$ is maintained throughout the experiment. **B.** Thermal printer recordings of representative points from plate A. PD, membrane potential.

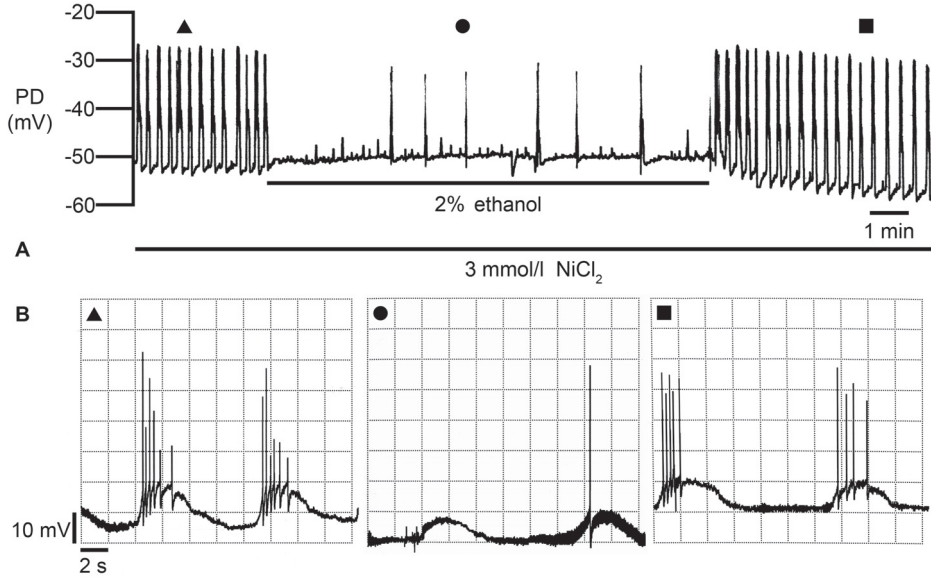

Figure 4. Effect of 2% ethanol on bursting of leech Retzius neuron. **A.** Trace from pen recorder. Upon application of ethanol frequency of bursting is diminished. Washout of ethanol restores bursting activity. Presence of $NiCl_2$ is maintained throughout the experiment. **B.** Thermal printer recordings of representative points from plate A. Ethanol reduces duration and amplitude of plateaus as well as number of spikes per plateau (● vs. ▲). PD, membrane potential.

48 plateaus) and the amplitude was diminished by 3.1 ± 0.4 mV from 9.7 ± 0.7 to 6.7 ± 0.6 mV ($p < 0.01$, $n = 48$ plateaus). During the application of EtOH-Ni-Ringer, action potentials rarely occurred during plateaus of depolarization, and average number of spikes per plateau was reduced from 5.8 ± 0.6 to 0.7 ± 0.2 ($p < 0.01$, $n = 30$ plateaus, Fig. 5). After washout with Ni-Ringer membrane potential fully recovered in all seven cells, and bursting activity resumed at near control values.

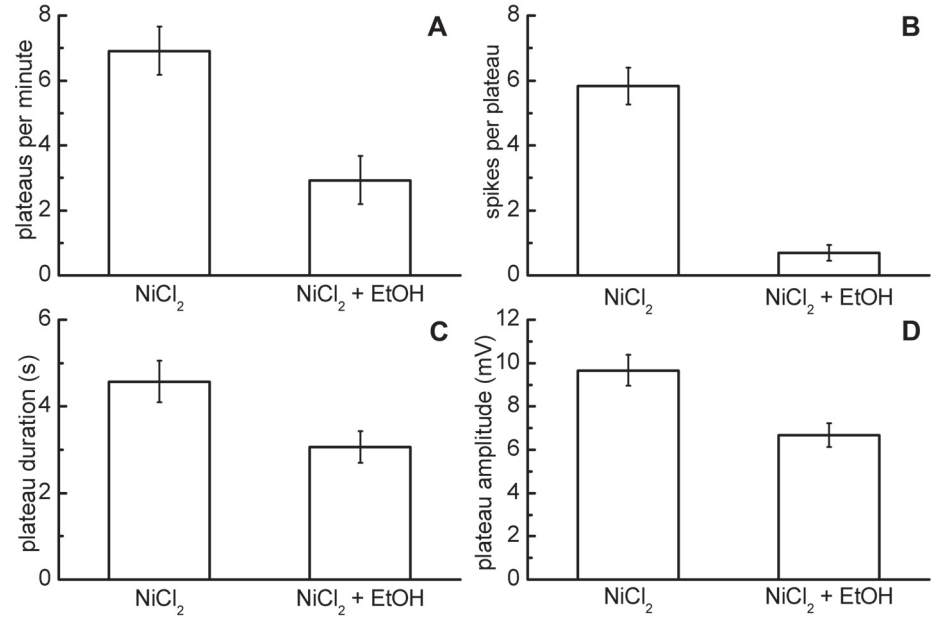

Figure 5. Graphic representation of effect of ethanol on frequency (A, $n = 7$ cells), number of spikes per plateau (B, $n = 30$ plateaus), plateau duration (C, $n = 48$ plateaus), and amplitude (D, $n = 48$ plateaus) of leech neuron bursting induced by $NiCl_2$. Error bars represent ± S.E.M.

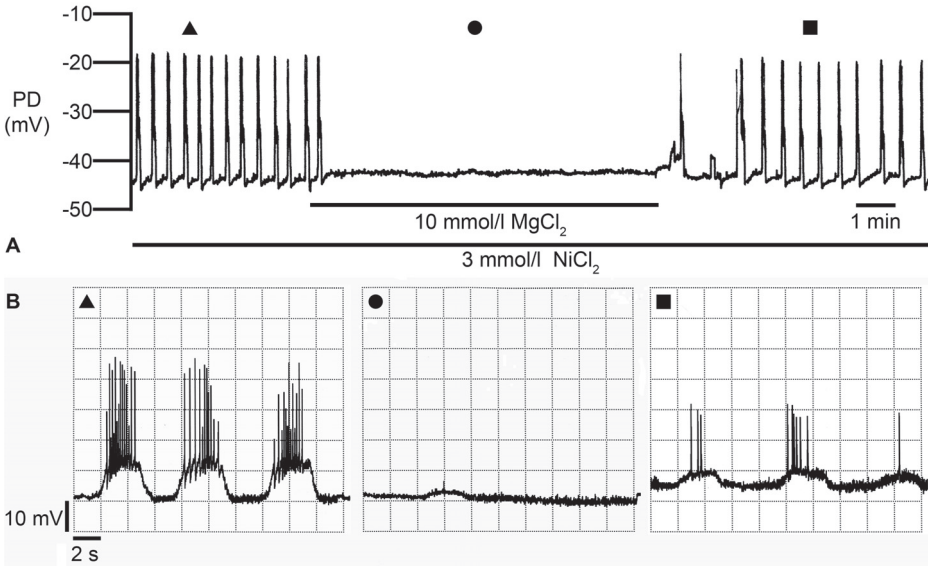

Figure 6. Effect of 10 mmol/l Mg^{2+} on bursting of leech Retzius neuron. **A.** Trace from pen recorder. Application of $MgCl_2$ apparently terminates the bursting. Washout of Mg^{2+} restores bursting activity. Presence of $NiCl_2$ is maintained throughout the experiment. **B.** Thermal printer recordings of representative points from plate A. Middle trace (●) reveals a plateau of depolarization diminished in duration and amplitude which does not elicit spikes. PD, membrane potential.

Effects of 10 mmol/l Mg^{2+} on Ni^{2+}-induced bursting activity

The effect of Mg^{2+} on Ni^{2+}-induced bursting activity is illustrated in Fig. 6. Oscillatory activity induced in Retzius cells in these experiments ($n = 7$, resting membrane potential –40.0 ± 3.4 mV) had the frequency of 5.7 ± 1.0 plateaus per minute. Superfusion with saline containing 10 mmol/l Mg^{2+} and 3 mmol/l Ni^{2+} (Mg-Ni-Ringer) almost completely eliminated the oscillatory activity of the neuron, reducing it to 0.2 ± 0.1 plateaus per minute ($p < 0.01$, Fig. 6A). Dur-

ing this period action potential generation was completely abolished, and plateaus of depolarization occurred only sporadically, i.e. we have recorded only four plateaus in all seven cells with combined duration of Mg-Ni-Ringer application spanning more than 70 min. The plateau duration was reduced from 3.9 ± 0.1 to 2.7 ± 0.3 s ($p < 0.01$, $n = 4$), and plateau amplitude was diminished by 4.5 ± 1.2 mV from 12.5 ± 0.3 to 8.0 ± 1.0 ($p < 0.05$, $n = 4$). One of the plateaus is shown in Fig. 6B. Washout with Ni-Ringer led to restoration of bursting activity.

Discussion

Na^+-dependent bursting and mechanism of burst initiation in H. sanguisuga

Many epileptic conditions are considered to be idiopathic and the related seizures of unknown origin. It does appear that different types of seizures are caused by differing mechanisms. Most membrane potential oscillations, burst firing, and seizure activity described to date are Ca^{2+}-dependent as they require influx of Ca^{2+} through voltage-gated Ca^{2+} channels. However, in some cases rhythmic bursting and seizures are produced in low or zero Ca^{2+} environments. It has been shown that in the avian Edinger Westphal nucleus in repetitively firing cells removal of extracellular calcium increases the frequency of action potential discharge (Fujii 1992), that low and zero Ca^{2+} solutions cause development of spontaneous, rhythmic and synchronous bursting discharges in rat hippocampal slices (Wang et al. 2004) and supraoptic nucleus neurons (Li and Hatton 1996), and also that low calcium levels can cause, or at least contribute to myoclonic seizures, infantile spasms, and seizures concurrent with Down syndrome (Thiel 2006).

Similar bursting activity can be induced in Retzius neurons of the leech *H. medicinalis* by block of Ca^{2+} currents with transitional metal ions such as Co^{2+}, Ni^{2+}, and others, Ni^{2+} being the most potent of them (Angstadt et al. 1998). The following mechanism has been proposed for the initiation of this Na^+-dependent bursting: block of voltage dependent Ca^{2+} influx suppresses Ca^{2+} dependent outward currents, namely calcium activated potassium current ($I_{K(Ca)}$) unmasking the effects of persistent inward Na^+ current resulting in a rapid depolarization, and supporting a maintained depolarizing plateau (Angstadt and Friesen 1991; Angstadt 1999).

In the present study we have used 3 mmol/l Ni^{2+} solution to induce bursting activity in the Retzius neurons of the leech *H. sanguisuga*. All characteristics of bursting in our model (bursting frequency, plateau duration and amplitude) are in keeping with results of Angstadt (1991) on *H. medicinalis*. Also, in our experiments the onset of oscillatory activity was preceded by membrane depolarization, a result consistent with block of tonic $I_{K(Ca)}$. Furthermore, we have shown that bursting of Retzius neurons in *H. sanguisuga* is also Na^+-dependent, as bursting is completely abolished in Na^+-free solution, and the pattern of changes induced by removal of Na^+ is virtually identical to that shown by Angstadt (1999). Therefore we conclude that the same mechanism as the one described by Angstadt for *H. medicinalis* leads to bursting initiation in *H. sanguisuga*.

Ethanol inhibits Ni^{2+}-induced bursting

Our results show that 2% ethanol suppresses Ni^{2+}-induced bursting activity of Retzius cells by lowering the frequency of bursts, and reducing the duration and amplitude of plateaus of depolarization, as well as the number of spikes per plateau. This is in agreement with pronounced antiepileptogenic and anticonvulsant effects of ethanol after acute application (Fischer and Kittner 1998).

The antiepileptogenic and anticonvulsant effects of ethanol could be explained by its effect on excitatory and inhibitory neurotransmission. There is substantial proof of ethanol action on N-methyl-D-aspartate (NMDA) (Kumari and Ticku 2000), non-NMDA, and γ-aminobutyric acid receptor function (Weight et al. 1992). It has also been shown that intoxicating concentrations of ethanol posses anticonvulsant activity through blockage of the NMDA receptor-mediated synaptic excitation (Gean 1992). However, this is an unlikely cause of ethanol suppression of bursting activity in our model, since Ni^{2+} solution used to induce bursting activity rapidly and completely blocks chemical synaptic transmission (Angstadt and Friesen 1991; Angstadt et al. 1998). Moreover, Retzius cells isolated in culture, and therefore deprived of synaptic inputs, exhibit rhythmic plateau potentials (Angstadt and Choo 1996).

A more plausible mechanism of ethanol suppression of bursting is block of voltage-dependent Na^+ channels. The acute intoxicating effects of ethanol have been widely attributed to its ability to block voltage-gated Na^+ channels (Fitzgerald and Nestler 1995). Ethanol exhibits significant inhibition of sodium current in Aplysia neurons (Camacho-Nasi and Treistman 1986), rat neurons (Harris and Bruno 1985a), and other preparations. This effect might be mediated by ethanol-induced membrane lipid disorder, secondarily affecting Na^+ channels (Harris and Bruno 1985b), or by direct action on the channel itself (Krylov et al. 2000).

Another possible avenue of ethanol effect on bursting is activation of $I_{K(Ca)}$. Studies performed in systems that likely represent relevant targets of alcohol actions in the body, such as the neurons involved in motor coordination, nociceptive and neuropeptide releasing-neurons, and growth-hormone releasing cells, at a variety of levels, and using a wide variety of approaches indicate that Ca^{2+}-activated K^+ channels

represent relevant targets in ethanol actions (Brodie et al. 2007). Open probability of Ca^{2+}-activated K^+ channels is increased 2–6 times depending on the concentration of ethanol, with a functional consequence of reduced firing frequency during ethanol application in dorsal root ganglia of rats (Gruss et al. 2001). The potentiation of Ca^{2+}-activated K^+channels by ethanol is a result of direct action on the channel itself (Dopico et al. 1999). Interestingly, this effect is especially pronounced when Ca^{2+} influx into the cell is minimal (Dopico et al. 1999), a case that corresponds very well to our model.

Since the mechanism of bursting in Retzius cells is directly related to the block of $I_{K(Ca)}$ and influx of Na^+ as described before, activation of Ca^{2+}-activated K^+ channels and block of voltage-gated Na^+ channels would clearly have detrimental effect on bursting activity.

Inhibition of Ni^{2+}-induced bursting by Mg^{2+}

Characteristic clinical features of hypomagnesemia and hypocalcemia include seizures (Baker and Worthley 2002). Further, the concentrations of cerebrospinal fluid-ionized magnesium in convulsive children is significantly lower than in non-convulsive children and is related to age-dependent changes in ionized calcium as well as decreased ionized magnesium in the developing brain (Miyamoto et al. 2004). Also, low Mg^{2+}-induced epilepsy in CA1 neurons of intact mice hippocampus is enhanced by Ca^{2+} channel blocker nifedipine (Derchansky et al. 2004). On the other hand, increasing Mg^{2+} levels suppress bursting induced by low Ca^{2+} in rat hippocampal slices (Wang et al. 2004).

Having in mind this apparent relationship of low Ca^{2+}, Mg^{2+}, and seizure generation, we have examined the effect of 10 mmol/l Mg^{2+} on Ni^{2+}-induced bursting of leech Retzius cell. Our results show that Mg^{2+} in concentration of 10 mmol/l almost completely blocks bursting activity, shortens the duration and decreases the amplitude of the plateaus in our model.

Proconvulsive and anticonvulsive effects of Mg^{2+} are mostly attributed to its modulatory action on NMDA receptors (Gean and Shinnick-Gallagher 1988). However, it has been demonstrated that block of NMDA receptors does not prevent all forms of Mg^{2+}-free-induced epileptiform activity in rats (Thomson and West 1986). Also, non-NMDA channel blocker CNQX is shown to have no effect on bursting activity induced by lowering extracellular Mg^{2+} concentration in rat hippocampal slices (Wang et al. 2004). Additionally, low Ca^{2+} and high Mg^{2+} solutions effectively block chemical synaptic transmission. Finally, as discussed before, chemical synaptic transmission bares no significance to bursting initiation in our model. Consequently, Mg^{2+} action on synaptic transmission does not seem to be an underlying mechanism of bursting termination in leech Retzius cells.

More probably, the effect of Mg^{2+} results from modulation of Na^+- and Ca^{2+}-dependent K^+ channels. Permeation across the Na^+ channels of rat hippocampal neurons is voltage- and concentration-dependently reduced by both intracellular (Lin et al. 1991), and extracellular Mg^{2+} (Sang and Meng 2002). The mechanism of this action is two-fold as Mg^{2+} changes the surface potential of the membrane and interferes with Na^+ permeation by competitively occupying Na^+ channels (Lin et al. 1991).

On the other hand, previous studies have shown a significant potentiation of Ca^{2+}-induced K^+ outward current (through Ca^{2+}-dependent K^+ channels) – after the addition of Mg^{2+} into the solution (McLarnon and Sawyer 1993). This potentiation was only observed if there had been Ca^{2+} present in the cell, but this agrees well with our results since we did not perform substitution of Ca^{2+} with Ni^{2+}, but rather an addition of Ni^{2+} to Ca^{2+} containing solution.

Thus, similarly to ethanol, there are two possible mechanisms of suppressive action of Mg^{2+} upon bursting, one affecting Na^+ channels, and the other affecting $I_{K(Ca)}$.

Concluding remarks

The chemical synapse-independent mechanism of ethanol and magnesium suppression of bursting activity by action on Na^+ channels and $I_{K(Ca)}$ proposed in this paper might have broader significance, pertaining not only to leeches, but mammalian neurons as well. It has been shown that phasic bursting in magnocellular neuropeptidergic cells is not dependent upon synaptically-mediated excitation (Hatton 1982), and that a low and zero Ca^{2+} mechanism of non-synaptic epileptogenesis exists in rat hippocampal neurons (Watson and Andrew 1995). Many anticonvulsant drugs target voltage-gated Na^+ channels (Remy et al. 2003), and Na^+ channel blocker YWI192 inhibits burst firing in CA1 neurons of animals with temporal lobe epilepsy (Jones et al. 2009). Lastly, increased excitability and rhythmic and synchronous bursting discharges that develop in rat hippocampal slices in solutions containing low Ca^{2+} are attributed to a Ca^{2+}-activated K^+ conductance (Martin et al. 2001).

It might also be noteworthy that there is an interplay of ethanol, Ca^{2+}, and Mg^{2+}. It is documented that ethanol consumption lowers both Ca^{2+} and Mg^{2+} levels in rabbits (Mahboob and Haleem 1988) and humans (Petroianu et al. 1991). Additionally, hypomagnesemia occurs in chronic alcoholism (Wu and Kenny 1996), and neurological signs which accompany ethanol ingestion are associated with rapid deficits in serum ionized magnesium (Altura and Altura 1999). It is not clear in which way combination of these disturbances leads to disease, but common sites of action postulated in this paper might shed some light on this problem as well.

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