

## Magnesium Effects on Nonsynaptic Epileptiform Activity in Leech Retzius Neurons\*

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The effects of  $Mg^{2+}$  on  $Ni^{2+}$ -induced epileptiform bursting activity and input membrane resistance during this activity of leech Retzius neurons were examined using intracellular recordings. To induce epileptiform activity, 3 mmol/l  $NiCl_2$  was added into superfusing Ringer (Ri) saline. To test for dose-dependence of the effects of  $Mg^{2+}$  on the induced epileptiform activity,  $MgCl_2$  was added in concentrations from 1 mmol/l to 20 mmol/l  $Mg^{2+}$  to the  $Ni^{2+}$ -containing Ri saline. Input membrane resistance (IMR) was measured in standard Ri,  $Ni^{2+}$  Ri and 20 mmol/l  $Mg^{2+}$   $Ni^{2+}$  Ri saline. Superfusion with  $Ni^{2+}$  Ri induced epileptiform bursting activity characterized by generation of paroxysmal depolarization shifts (PDSs). Parameters of epileptiform activity including PDS frequency, PDS duration, PDS amplitude and the number of spikes/PDS were measured. Magnesium suppressed  $Ni^{2+}$ -induced epileptiform activity, significantly reducing values of all parameters observed in a concentration-dependent manner. The highest concentration applied of 20 mmol/l  $Mg^{2+}$  completely eliminated epileptiform activity. To test for the effect of  $Mg^{2+}$  on membrane conductance during bursting, IMR was measured. Magnesium significantly increased IMR during bursting suppression.

Key words: Nonsynaptic bursting, input membrane resistance, leech Retzius neuron, magnesium.

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Paroxysmal depolarization shifts (PDSs) are prolonged, large amplitude membrane depolarizations superimposed by high-frequency bursts of action potentials (PRINCE 1978). They represent a major cellular event underlying epileptogenesis. The mechanisms of PDS generation have been studied on various models both *in vivo* and *in vitro* (DICHTER & AYALA 1987; STRAFSTROM 2007). In leech Retzius neurons (LRNs) the generation of PDSs can readily be provoked by several exogenous agents such as penicillin, bemegride and phenobarbital (PRICHARD 1972), neutral red (LENT & FRAZER 1977), various pyrethroids (LEAKE 1982), and FMRF-amide (SAHLEY *et al.* 1993). Rhythmi-

cal bursting activity can also be induced in LRNs by lowering the concentration of  $Cl^-$  or  $Ca^{2+}$  in bath media (YANG & LENT 1983; BECK *et al.* 2001), or by replacement of external  $Ca^{2+}$  by  $Ba^{2+}$ ,  $Co^{2+}$  and other inorganic  $Ca^{2+}$  channel blockers, such as  $Ni^{2+}$ ,  $Mn^{2+}$ ,  $Cd^{2+}$ ,  $Zn^{2+}$  and  $La^{3+}$  (YANG & LENT 1983; DEAN & LEAKE 1988; ANGSTADT *et al.* 1998). Therefore, LRN represents a valuable model for elucidating the cellular mechanisms of epileptogenesis (YANG & LENT 1983; ANGSTADT & FRIESEN 1991), as well as for the investigation of the effects and mechanisms of action of various antiepileptic drugs.

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Magnesium is one of these substances as it has an anticonvulsive effect in a clinical setting, and is used for control of seizures in preeclampsia and eclampsia (SIBAI 2005). *In vitro* and *in vivo* experiments have provided additional evidence for the anticonvulsant effect of  $Mg^{2+}$  (STANDLEY *et al.* 1995; WANG *et al.* 2004), including the inhibitory effect on  $Ni^{2+}$ -induced  $Na^+$ -dependent epileptiform activity on our model (PATHAK *et al.* 2009). The present study was performed in order to further examine the antiepileptic properties of  $Mg^{2+}$  and to test for the underlying mechanism. To this end we have evaluated the dose-dependency of  $Mg^{2+}$  suppression of  $Ni^{2+}$ -induced epileptiform activity of Retzius neurons of the leech *Haemopsis sanguisuga*. Furthermore, input membrane resistance (IMR) was examined prior to and during the suppression of bursting by  $Mg^{2+}$ , as a measure of the alterations of the membrane conductance induced by  $Mg^{2+}$ . Our results show that  $Mg^{2+}$  ions suppress  $Ni^{2+}$ -induced epileptiform activity in a dose-dependent manner and increase IMR during bursting suppression.

## Material and Methods

The experiments were performed at room temperature (22–25°C) on Retzius neurons in the isolated segmental ganglia of the ventral nerve cord of the leech *Haemopsis 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 a 2.5 ml plastic chamber with leech Ringer solution and fixed by means of fine steel clips. The plastic chamber was then placed in a grounded Faraday's cage mounted on a fixed table in a manner that prevents vibrations. Identification and penetration of the cells were performed in the cage under a stereomicroscope. Retzius neurons were identified by their position on the ventral surface of the ganglion, their size and their bioelectrical properties. To change the solution, the chamber was flushed with a volume of fluid at least 5 times that of the chamber volume. The superfusion was usually completed in 10–15 s.

### Electrophysiological recordings

The membrane potential was recorded using standard single-barrel glass microelectrodes. Micropipettes were pulled from glass capillaries with internal filament (Harvard Apparatus GC150F-10, UK) on a vertical puller (Narishige, Tokyo, Japan) and then filled with 3 mol/l KCl shortly after being pulled. The microelectrode resistance was 20–35 M $\Omega$ . The recordings were amplified using a high

input impedance amplifier (model 1090, Winston Electronics, San Francisco, CA, USA;  $R=10^9 \Omega$ ). Microelectrodes were connected to the amplifier via a Ag-AgCl wire. The ground electrode was a Ag-AgCl wire in a separate chamber filled with Ringer solution connected to the experimental chamber by a 3 mol/l KCl 3% agar bridge. For IMR measurements of the directly polarized membrane, a high input impedance bridge amplifier (model 1090, bridge unit BR1, Winston Electronics, San Francisco, CA, USA) was used to inject a current through the recording microelectrode. Rectangular hyperpolarizing pulses (0.3–2.0 nA, 500 ms duration, applied at a frequency of 0.1–0.2 Hz) were delivered using an S48 dual output square-pulse stimulator and SIU5 stimulus isolation unit (both Grass Instruments, Quincy, MA, USA). The amplitudes of the evoked electrotonic potentials were used to calculate IMR of LRNs. The recordings were displayed on a two channel oscilloscope (Hameg, Frankfurt am Main, Germany) and permanently recorded on a pen recorder (Linseis, Selb, Germany), and a thermal graphic printer (Hameg, Frankfurt am Main, Germany).

### Solutions

The standard Ringer solution (Ri) used in these experiments had the following composition (in mmol/l): NaCl 115.5, KCl 4,  $CaCl_2$  2,  $Na_2HPO_4$  1.2,  $NaH_2PO_4$  0.3 (pH=7.2). In the  $Ni^{2+}$ -containing solution ( $Ni^{2+}$  Ri) 3 mmol/l  $NiCl_2$  was added. To make a series of  $Ni^{2+}$  Ri solutions containing  $Mg^{2+}$  ( $Mg^{2+}$   $Ni^{2+}$  Ri) with increasing concentrations of  $Mg^{2+}$  (1 mmol/l, 3 mmol/l, 7 mmol/l, 10 mmol/l and 20 mmol/l), the appropriate amount of 1 mol/l  $MgCl_2$  stock solution was added to  $Ni^{2+}$  Ri saline and an adequate amount of NaCl reduced for osmotic correction.

### Data analysis

All results are expressed as means  $\pm$  SEM, with *n* indicating the number of experiments. All fitting procedures were based on the least squares method. Comparisons between mean values were made using the two-tailed paired Student's *t*-test; *P* values of less than 0.05 were considered significant.

## Results

### Epileptiform activity induced by nickel

In standard Ri solution LRNs had resting membrane potential (RMP) of  $-43.17 \pm 0.79$  mV ( $n=39$ ) and spontaneously fired single action potentials. Superfusion of isolated ganglia with Ri solution containing  $Ca^{2+}$  channel blocker nickel (3 mmol/l

NiCl<sub>2</sub>) induced epileptiform activity. This activity was oscillatory and rhythmic, and characterized by repetitive generation of PDSs – large amplitude plateau-like waves of depolarization lasting several seconds and superimposed by bursts of action potentials. The PDSs developed gradually over 2-7 minutes increasing in frequency, duration, amplitude and number of spikes/PDS, eventually reaching a final level sustained throughout Ni<sup>2+</sup> Ri exposure (Fig. 1). During stabilized Ni<sup>2+</sup>-induced bursting, RMP was  $-41.91 \pm 1.02$  mV, PDS frequency was  $5.92 \pm 0.28$  min<sup>-1</sup>, PDS amplitude  $12.35 \pm 0.43$  mV and PDS duration  $4.81 \pm 0.14$  s, with an average of  $7.17 \pm 0.50$  spikes/PDS. Paroxysmal depolarization shifts ended by rapid repolarizations and were followed by inter-PDS intervals which consisted of slowly depolarizing ramp potentials.

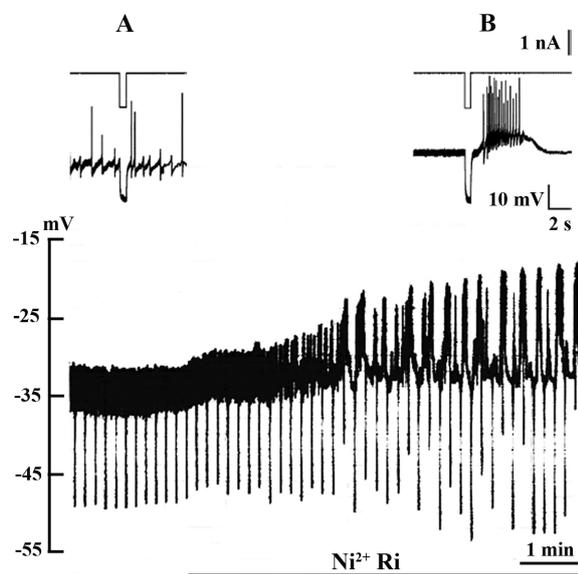


Fig. 1. Application of Ni<sup>2+</sup> Ri induces bursting activity and increases IMR of leech Retzius neurons. The lower trace is from a mechanical pen recorder and the upper traces represent recordings of electrotonic potentials evoked in response to hyperpolarizing current pulses in standard Ri solution (A), and during bursting activity in Ni<sup>2+</sup> Ri solution (B).

#### Effect of 3 mmol/l Ni<sup>2+</sup> on input membrane resistance

In this group of experiments IMR was measured between single action potentials during normal LRN activity in standard Ri, and between PDSs during oscillatory activity in Ni<sup>2+</sup> Ri. In standard leech Ri, IMR was  $8.93 \pm 0.82$  M $\Omega$  (n=9). Application of Ni<sup>2+</sup> Ri led to a highly significant increase of IMR to  $12.33 \pm 0.65$  M $\Omega$  (P<0.001), denoting that Ni<sup>2+</sup> acts to decrease membrane conductance (Fig. 1).

#### Effects of Mg<sup>2+</sup> on Ni<sup>2+</sup>-induced epileptiform activity

To study the effects of rising concentrations of Mg<sup>2+</sup> on Ni<sup>2+</sup>-induced epileptiform activity, we applied Ni<sup>2+</sup> Ri saline containing Mg<sup>2+</sup> in five different concentrations: 1 mmol/l, 3 mmol/l, 7 mmol/l, 10 mmol/l and 20 mmol/l. To the best of our knowledge, dose-dependency of the suppressive effect of Mg<sup>2+</sup> on epileptiform activity has previously not been reported. In all cases Mg<sup>2+</sup> Ni<sup>2+</sup> Ri saline was applied after bursting had stabilized. Parameters of epileptiform activity including PDS frequency, PDS duration and PDS amplitude, as well as the number of spikes/PDS were measured prior to and during superfusion with Mg<sup>2+</sup> Ni<sup>2+</sup> Ri. Each of the applied Mg<sup>2+</sup> concentrations significantly reduced the values of all observed bursting parameters (Table 1). The highest concentration of 20 mmol/l Mg<sup>2+</sup> rapidly and completely eliminated epileptiform activity for as long as the presence of Mg<sup>2+</sup> was maintained in the bath. Washout with Ni<sup>2+</sup> Ri restored bursting activity. In some of these trials the Mg<sup>2+</sup> Ni<sup>2+</sup> Ri solution was reapplied after bursting recovery with Ni<sup>2+</sup>, and bursting suppression always reoccurred in the presence of Mg<sup>2+</sup>.

#### Dose-response analysis

Since overall bursting suppression by Mg<sup>2+</sup> on our model increased with increasing Mg<sup>2+</sup> concentration, we carried out a formal quantitative and graphical analysis of dose dependency. The values of each of the four PDS parameters were plotted

Table 1

#### Dose-dependent bursting suppression by Mg<sup>2+</sup>.

Mg <sup>2+</sup> concentration (mmol/l)	PDS frequency (%)	PDS duration (%)	PDS amplitude (%)	Spikes/PDS (%)	N
1	70.47±5.94 P<0.05	86.79±3.21 P<0.05	83.84±6.30 P<0.05	79.74±11.76 P<0.05	5
3	57.89±5.26 P<0.001	86.20±5.20 P<0.05	74.46±15.26 P<0.05	73.72±12.54 P<0.05	7
7	48.31±8.46 P<0.001	75.62±5.99 P<0.01	75.00±5.38 P<0.01	63.04±11.15 P<0.05	8
10	19.56±7.65 P<0.001	65.27±10.98 P<0.01	49.52±10.35 P=0.001	45.50±9.92 P<0.05	11
20	0.00±0.00 P<0.001	0.00±0.00 P<0.001	0.00±0.00 P<0.001	0.00±0.00 P<0.001	8

Data shown as mean±SEM; PDS – paroxysmal depolarization shift; N – number of cells.

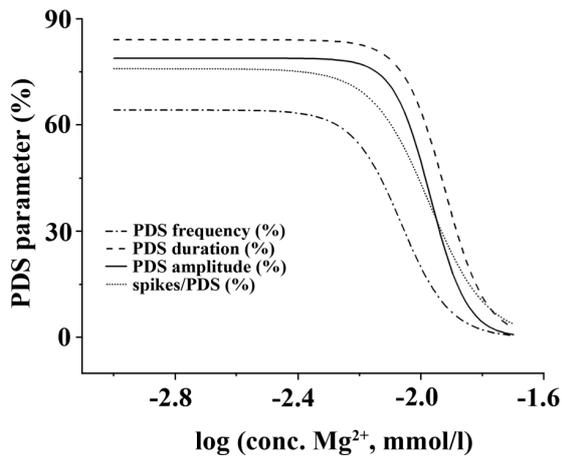


Fig. 2. Dose-response curves for the effects of  $Mg^{2+}$  on parameters of  $Ni^{2+}$ -induced epileptiform activity of leech Retzius neurons. Control values were obtained in the absence of  $Mg^{2+}$ . Experimental values are presented as % of control. Log-linear axes. Data points and error bars omitted for clarity. Magnesium reduces values of bursting parameters in a dose-dependent manner. conc. – concentration.

against the applied concentration of  $Mg^{2+}$  (Fig. 2). Analytical results and multiple data plots suggest a dose-dependent nature of  $Mg^{2+}$  block. The half maximum inhibitory response concentration ( $IC_{50}$ ) was determined for each curve. For all series of data points, fitted curves showed very similar numeric  $IC_{50}$  values between 8.61 and 11.91 mmol/l  $Mg^{2+}$ .

Effect of 20 mmol/l  $Mg^{2+}$  on input membrane resistance during  $Ni^{2+}$ -induced epileptiform activity

Since a concentration of 20 mmol/l  $Mg^{2+}$  caused a complete bursting blockade, a set of experiments

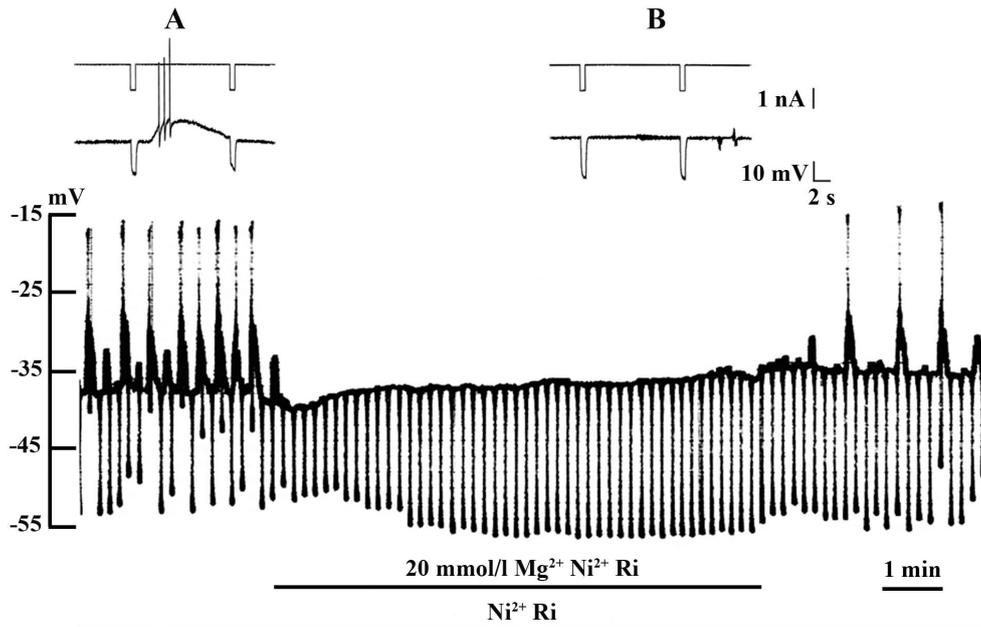


Fig. 3. Effect of 20 mmol/l  $Mg^{2+}$  on input membrane resistance (IMR) of leech Retzius neurons during bursting suppression. Pen recorder trace (below) with recordings of representative electrotonic potentials evoked in response to hyperpolarizing current pulses (above) in  $Ni^{2+}$  Ri (A), and during  $Mg^{2+}$ -induced bursting suppression (B). Application of  $Mg^{2+}$   $Ni^{2+}$  Ri increases IMR. Presence of  $Ni^{2+}$  is maintained throughout the experiment.

was performed to measure and compare IMR in  $Ni^{2+}$  Ri and in 20 mmol/l  $Mg^{2+}$   $Ni^{2+}$  Ri solution. The average value of RMP of LRNs in these experiments was  $-38.96 \pm 1.55$  mV. After induction and stabilization of epileptiform activity, IMR in  $Ni^{2+}$  Ri was measured to be  $13.00 \pm 1.57$  M $\Omega$ . Superfusion with 20 mmol/l  $Mg^{2+}$   $Ni^{2+}$  Ri significantly increased IMR to  $15.38 \pm 0.84$  M $\Omega$  ( $n=6$ ,  $P<0.05$ , Fig. 3). Therefore, the presence of  $Mg^{2+}$  in the solution caused a further increase in IMR when compared to the effect of  $Ni^{2+}$  alone, as shown in Fig. 4.

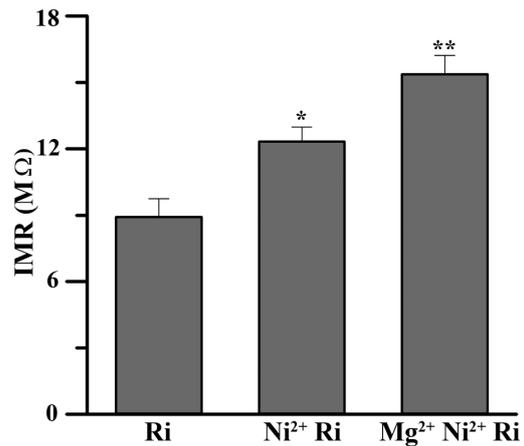


Fig. 4. Graph illustrating the effects of  $Ni^{2+}$  Ri and  $Mg^{2+}$   $Ni^{2+}$  Ri on input membrane resistance (IMR) of leech Retzius neurons. Nickel increases IMR. Introducing magnesium still further increases IMR. Values are averages, whereas error bars represent SEM. \* significant relative to standard Ri solution ( $P<0.001$ ), \*\* significant relative to  $Ni^{2+}$  Ri solution ( $P<0.05$ ).

## Discussion

The present study uses the Ni<sup>2+</sup>-induced epileptiform activity model of the LRN to test for the effects of Mg<sup>2+</sup> saline on bursting, and also to investigate the effect of high Mg<sup>2+</sup> on membrane conductance during bursting through the measurement of IMR.

In our model, the application of Mg<sup>2+</sup> solution led to a significant reduction of all bursting parameters in a concentration-dependent manner, and the highest concentration applied of 20 mmol/l Mg<sup>2+</sup> rapidly and completely eliminated epileptiform activity. This is in good agreement with the results of BORGES and GÜCER (1978) who have reported that MgSO<sub>4</sub> is able to suppress epileptic neuronal activity induced by topical application of penicillin on motor cortex in cats, dogs and primates. STANDLEY *et al.* (1995) also found that intraperitoneal injection of MgSO<sub>4</sub> significantly shortens seizure duration in hippocampal-kindled rats. On the other hand, lowering of extracellular Mg<sup>2+</sup> concentration leads to epileptiform activity in many parts of the central nervous system (WALTHER *et al.* 1986). All of these results suggest that Mg<sup>2+</sup> can be used as a therapeutic agent in some forms of epilepsy (JAMES 2010).

However, in spite of the increasing evidence showing magnesium's antiepileptic potential both clinically and experimentally, the exact mechanisms and sites of action still remain controversial and largely hypothetical (HUNTER & GIBBINS 2011). Therefore, we have tested the effect of high Mg<sup>2+</sup> on membrane conductance during bursting. We report for the first time that Mg<sup>2+</sup> significantly increases the input membrane resistance, i.e. reduces the membrane conductivity during epileptiform activity. Reduction in membrane conductivity could be the result of multiple mechanisms. Some of the possibilities will be discussed in more detail.

One possible mechanism for IMR increase is blockade of NMDA receptors by Mg<sup>2+</sup>. The nickel solution used to induce epileptiform activity in our model rapidly and completely blocks chemical synaptic transmission via Ca<sup>2+</sup> channel blockade (ANGSTADT & FRIESEN 1991; ANGSTADT *et al.* 1998). Therefore NMDA receptor blockade by Mg<sup>2+</sup> seems to be an unlikely cause of membrane conductance reduction and bursting suppression by Mg<sup>2+</sup> on our model. The direct effects of Mg<sup>2+</sup> on ion channels implicated in Ni<sup>2+</sup>-induced bursting are a more probable mechanism of magnesium's effect.

The mechanism of Ni<sup>2+</sup>-induced bursting in LRNs in *H. medicinalis* and *H. sanguisuga* is Na<sup>+</sup>-dependent and closely related to the suppression of tonic Ca<sup>2+</sup>-activated K<sup>+</sup> current (I<sub>K(Ca)</sub>) and a consequential prevalence of a persistent Na<sup>+</sup> current (I<sub>Na(p)</sub>) (ANGSTADT & FRIESEN 1991; PATHAK *et al.*

2009). Taken together, this previously shown Na<sup>+</sup>-dependency of the induced bursting and a decrease by Mg<sup>2+</sup> in membrane conductivity during bursting suppression demonstrated here, lead to a possibility of Mg<sup>2+</sup> action via Na<sup>+</sup> channels. This is in line with the finding that in dissociated CA1 rat hippocampal neurons, extracellularly applied MgSO<sub>4</sub> causes a concentration-dependent and voltage-dependent reversible decrease of Na<sup>+</sup> currents (SANG & MENG 2002). It is noteworthy that the dose-dependency of our results is in keeping with the dose-dependent decrease of Na<sup>+</sup> currents by Mg<sup>2+</sup> reported by SANG and MENG.

The reduction of Na<sup>+</sup> currents by Mg<sup>2+</sup> could also be attributed to the membrane stabilizing effect of Mg<sup>2+</sup> ions (FRANKENHAEUSER & HODGKIN 1957). Stabilizing properties of Mg<sup>2+</sup> ions are mostly due to the increase in surface charge screening which affects the voltage sensor of the voltage-gated Na<sup>+</sup> channels changing their gating properties (SOMJEN 2004). It has been shown that an increase of the extracellular Mg<sup>2+</sup> concentration leads to an activation curve shift towards positive potential and inhibits the activation process in acutely isolated CA1 neurons and cerebellar granular cells (LIN *et al.* 1991; SANG & MENG 2002). Interestingly, LIN *et al.* (1991) report that Mg<sup>2+</sup> had no effect on activation kinetics in doses equal to or smaller than 7 mmol/l. Our dose-response curves also show that 7 mmol/l seems to be a cut-off point after which the curve becomes much steeper.

On the other hand, Ca<sup>2+</sup>-activated K<sup>+</sup> (K<sub>Ca</sub>) conductance is critically implicated in epileptogenesis in our model, as previously described (PATHAK *et al.* 2009). However, Mg<sup>2+</sup> is known to block the majority of known K<sup>+</sup> channels (BARA *et al.* 1993), and the results concerning the Mg<sup>2+</sup> effect on K<sub>Ca</sub> channel permeability are contradictory (FERGUSON 1991). Furthermore, activation of K<sub>Ca</sub> channels should lead to IMR reduction, whereas we have shown an IMR increase.

In conclusion, Mg<sup>2+</sup> ions suppress Ni<sup>2+</sup>-induced epileptiform activity of LRNs in a dose-dependent manner and increase IMR during bursting suppression.

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