Journal of Elementology ISSN 1644-2296

Stanojević M., Lopicic S., Spasic S., Vukovic I., Nedeljkov V., Prostran M. 2016. *Effects of high extracellular magnesium on electrophysiological properties of membranes of Retzius neurons in leech Haemopis sanguisuga*. J. Elem., 21(1): 221-230. DOI: 10.5601/jelem.2015.20.1.887

EFFECTS OF HIGH EXTRACELLULAR MAGNESIUM ON ELECTROPHYSIOLOGICAL PROPERTIES OF MEMBRANES OF RETZIUS NEURONS IN LEECH *HAEMOPIS SANGUISUGA**

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Abstract

Magnesium is a bioessential cation with an important role in the function of excitable cells both in health and disease. Magnesium has therapeutic use as an anticonvulsant, anaesthetic, analgesic and antiarrhythmic agent. The aim of this work was to examine in detail the effects of high extracellular Mg^{2+} on the nerve cell membrane using classical electrophysiology techniques. The experiments were conducted on Retzius neurons in the isolated segmental ganglia of the leech *Haemopis sanguisuga*. Intracellular recording of membrane potential and electrical activity, as well as current clamp experiments to examine membrane input resistance and excitability, were performed prior to and during application of 20 mmol dm^{-3} $MgCl_2$. The paper presents our findings on the effects of high Mg^{2+} on basic electrophysiological properties and activity of Retzius cells. Depolarization of the membrane potential, a decrease in the frequency of spontaneous activity, an increase in threshold potential, a decrease in cell excitability and an increase in input membrane resistance were found following an application of high Mg^{2+} solution. The underlying mechanisms of the overall suppressive action of Mg^{2+} on our cell model are discussed to be multiple Mg^{2+} effects on different ion channel conductances, with a possibly dominant blockade of Na^+ channels and a probable modulation of activity of Ca^{2+}-activated K^+ channels by Mg^{2+}.

Keywords: spontaneous activity, excitability, input membrane resistance, leech Retzius neuron, magnesium.

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* This work was supported by the Ministry of Education, Science and Technological Development of Republic of Serbia, grant number 175023.

INTRODUCTION

Magnesium is the eighth most common element in the Earth's crust and a bioessential mineral (Jahnen-Dechent, Ketteler 2012). In mammalians, it is the second most abundant intracellular and the fourth most abundant extracellular cation. Biologically, Mg^{2+} is quite versatile as it serves numerous structural and dynamic functions in the cell (Wolf, Trapani 2008). Despite both seasonal and local variations of the magnesium content in the environment and living organisms, biogeochemical surveys in many countries consistently report magnesium deficiency in plants and animals, presumably due to the leaching of magnesium from soil caused by acid rains (Jović 1999). Furthermore, in humans, magnesium depletion is common since the dietary intake of processed foods with low magnesium content is increasing. In clinical practice, hypomagnesemia is now generally considered the most common unidentified electrolyte disbalance.

Magnesium also has the potential of a pharmacological agent with diverse clinical applications (Akhtar et al. 2011). Infusions of Mg^{2+} salts are used in a variety of conditions: for eclamptic (James 2010) and porphyric convulsions (Sadeh et al. 1991), in the treatment of torsades de pointes tachycardia (Fawcett et al. 1999), and as adjuvant in anaesthesia (James 2009) and analgesia (Širvinskas, Laurinaitis 2002). However, despite its effective and multidisciplinary clinical use, the mechanisms underlying many salutary effects of simple Mg^{2+} ion are still a matter of controversy.

Magnesium level in the cerebrospinal fluid (CSF) confirms its role as an important regulator of nerve cell excitability. Physiologically, Mg^{2+} is the only cation besides H^+ found in a higher concentration in the CSF than in blood (Somjen 2004). Pathophysiologically, altered ionized Mg^{2+} levels are found in serum in a range of neurological conditions, e.g. in stroke patients, patients with brain trauma and migraine, or in both serum and the CSF, e.g. in epilepsy (Miyamoto et al. 2004, Haensch 2010), suggesting that Mg^{2+} disbalance may be a relevant factor in the pathogenesis of neuronal damage and dysfunction. Moreover, Furukawa et al. (2009) reported that Mg^{2+} requirement in the rat brain is higher in the hippocampus than in the cortex, suggesting regional differences in sensitivity of neurons to extracellular Mg^{2+} (Mg^{2+}_e).

Experimental studies of Mg^{2+} are regaining increased attention. Since further insight into mechanisms of Mg^{2+} action on excitable membranes is needed, we performed the experiments in order to examine the effects of Mg^{2+} on neuronal basic intrinsic membrane properties and activity. The effects of high Mg^{2+} on the resting membrane potential (RMP), spontaneous firing pattern, threshold potential, as well as the evoked activity and input membrane resistance (IMR) of the leech Retzius neurons (LRNs) were tested prior to and after the exposure to Mg^{2+}-containing saline.

MATERIAL AND METHODS

The experiments were performed at room temperature (22-25°C) on Retzius neurons in the isolated segmental ventral nerve cord ganglia of the leech *Haemopis sanguisuga*. The method of dissection has been previously described (BELESLIN 1971) and complies with institutional research council guidelines. Dissected ganglia were immediately transferred to a 2.5 cm^3 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, size and 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, Japan) and filled with 3 mol dm^{-3} KCl shortly after being pulled. The microelectrode resistance was 20-35 MΩ. The ground electrode was a Ag-AgCl wire in a separate chamber filled with Ringer solution connected to the experimental chamber by 3 mol dm^{-3} KCl – 3% agar bridge. Microelectrodes were connected to an amplifier *via* a Ag-AgCl wire. A high input impedance bridge amplifier (model 1090, bridge unit BR1, Winston Electronics, USA; R = 10^9 Ω) was used to amplify the recordings and to inject a current through the recording microelectrode. To examine cell excitability and measure the IMR of the directly polarized membrane, constant rectangular current pulses were delivered using an S48 dual output square-pulse stimulator an SIU5 stimulus isolation unit (both Grass Instruments, USA). The number of action potentials (APs) evoked by depolarizing pulses (0.6-1.9 nA, 500 ms, 0.1 Hz) was used as a measure of cell membrane excitability. The AP threshold was measured at the point where the fastest rising phase of the AP started. The amplitudes of the electrotonic potentials evoked by hyperpolarizing pulses (0.3-2.0 nA, 500 ms, 0.1-0.2 Hz) were used to calculate IMR of LRNs. The recordings were displayed on a two channel oscilloscope (Hameg, Germany) and permanently recorded on a pen recorder (Linseis, Germany) and a thermal graphic printer (Hameg, Germany).

Solutions

The standard Ringer (Ri) solution used in these experiments had the following composition (mmol dm^{-3}): NaCl – 115.5, KCl – 4, $CaCl_2$ – 2, Na_2HPO_4 – 1.2, NaH_2PO_4 – 0.3 (pH = 7.2). To make Mg^{2+}-containing solution

(Mg^{2+} Ri), 20 mmol dm^{-3} $MgCl_2$ was added to standard Ri and an adequate amount of NaCl reduced for osmotic correction.

Data analysis

All data are expressed as means±standard error of mean (SEM), with n indicating the number of trials. Comparisons between mean values were done using two-tailed paired Student's t–test, with p values less than 0.05 considered statistically significant, and those less than 0.01 - highly statistically significant.

RESULTS AND DISCUSSION

Effects of 20 mmol dm^{-3} Mg^{2+} on membrane potential and spontaneous activity

Experiments were performed to examine the effects of high Mg^{2+} on RMP and spontaneous firing pattern of LRNs. In standard Ri solution LRNs had stable RMP of –42.76 ±1.24 mV and spontaneously fired APs at a low rate of 0.30±0.07 Hz (n = 7, Figure 1*a*). Superfusion with 20 mmol dm^{-3} Mg^{2+} Ri caused a slight initial hyperpolarization soon followed by a membrane potential depolarization by 4.20±0.49 mV to an average value of –38.56±1.19 mV

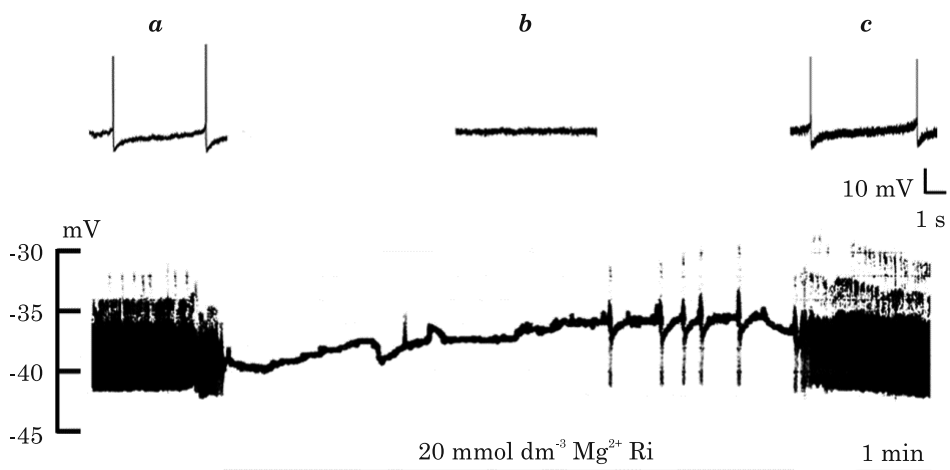

Fig. 1. Effects of 20 mmol dm^{-3} Mg^{2+} on membrane potential and spontaneous activity of the leech Retzius neuron. Lower trace is from a mechanical pen recorder and upper traces are oscilloscope screen recordings of representative points: *a* – in standard Ri solution Retzius neuron has a stable resting membrane potential and spontaneously fires action potentials (APs) at a low rate, *b* – application of 20 mmol dm^{-3} Mg^{2+} Ri saline causes small membrane potential depolarization and inhibition of AP discharge, *c* – after Ri solution is reapplied both membrane potential and frequency of spontaneous activity recover. Magnesium slightly depolarizes the cell and suppresses spontaneous activity

($p < 0.01$). Concomitently with the depolarization, spontaneous firing frequency was reduced to 0.03±0.01 Hz ($p < 0.01$). Figure 1*b* shows Mg^{2+}-induced membrane depolarization and a substantial decrease in the spontaneous firing frequency. After Mg^{2+} washout, LRNs quickly recovered to previous levels of membrane potential and impulse firing rate (Figure 1*c*).

Former reported findings concerning the effects of Mg^{2+} on neuronal RMP differ. Del Castillo and Engbaek (1954) observed that Ri solution containing 15 mmol dm^{-3} Mg^{2+} did not significantly alter the muscle fibre RMP. Similarly, Kato et al. (1968) and Kelly et al. (1969) found little or no change in membrane potential of cat cortical neurons with Mg^{2+} applied iontophoreticaly. Opposingly, Dribben et al. (2010) report of a minor depolarization of dissociated rat hippocampal neurons of approximately +5.2 mV shift in mean RMP in 11 mmol dm^{-3} Mg^{2+}. We have observed in our experiments that in LRNs a concentration of 20 mmol dm^{-3} Mg^{2+} produced an alteration of membrane potential typically comprising two phases: a transient slight initial hyperpolarization immediately following Mg^{2+} exposure, soon to give way to a slow small membrane depolarization that remained stable and sustained throughout Mg^{2+} exposure. High extracellular Mg^{2+} was applied in order to effectively block chemical synaptic transmission (Erulkar et al. 1974, Somjen 2004) and enable us to examine the nonsynaptic effects of Mg^{2+}.

In LRNs of *H. medicinalis* the intracellular Mg^{2+} concentration ($[Mg^{2+}]_i$) is regulated by an Na^+/Mg^{2+} antiport system. Simultaneous measurements of $[Mg^{2+}]_i$, intracellular sodium concentration ($[Na^+]_i$) and cell membrane potential show that an increase in extracellular magnesium concentration ($[Mg^{2+}]_o$) results in $[Mg^{2+}]_i$ increase, a parallel $[Na^+]_i$ decrease, and a consecutive membrane depolarization, due to the electrogenic nature of $1Na^+/1Mg^{2+}$ antiport system (Günzel, Schlue 1996). This is in accordance with our results of a minor Mg^{2+}-induced membrane depolarization of LRNs of *H. sanguisuga*.

Effect of 20 mmol dm^{-3} Mg^{2+} on threshold potential and evoked activity

Next, the effects of high Mg^{2+} on direct excitability of LRN membrane were tested. Injecting the constant depolarizing current pulses to the cells generated a train of spikes. The differences in threshold potential and the number of evoked responses prior to and upon the application of Mg^{2+} saline were measured (Figure 2). In standard Ri, threshold potential for AP generation in LRNs was -31.96±1.40 mV ($n = 5$). Superfusion with Mg^{2+} Ri increased AP threshold to -28.30±2.45 mV ($p < 0.05$). Intracellular depolarizing pulses generated an average number of 4.29±0.53 APs in standard Ri (Figure 2*a*), while in high Mg^{2+} Ri they elicited 1.57±0.52 APs ($n = 7$, $p < 0.01$, Figure 2*b*). An increase in AP threshold and a reduced number of evoked spikes indicate a significant decrease in cell excitability in high Mg^{2+}. In 2 cells the suppression of spike initiation was so pronounced that we were unable to evoke APs in the presence of Mg^{2+}. Superfusion with standard Ri solution reverses the effect (Figure 2*c*).

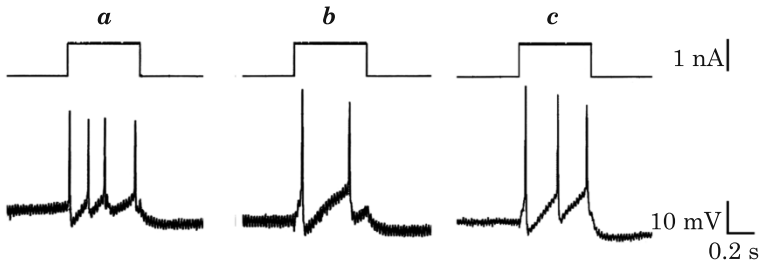

Fig. 2. Effect of 20 mmol dm^{-3} Mg^{2+} on excitability of the leech Retzius neuron. Above: current traces, below: voltage traces. Oscilloscope screen recordings: *a* – in control Ri solution, *b* – in 20 mmol dm^{-3} Mg^{2+} Ri, and *c* – after washout of Mg^{2+} with standard Ri. Activity evoked by depolarizing current pulses decreases and threshold potential increases in the presence of Mg^{2+}

A number of authors observed that Mg^{2+} can depress neuronal firing on other *in vitro* models: cat cortical and spinal neurons (Kato et al. 1968, Somjen, Kato 1968), isolated frog spinal cord (Erulkar et al. 1974), mouse brain stem neurons (Wojtowicz et al. 1977), and rat hippocampal neurons (Dribben et al. 2010). Our findings of nearly complete silencing of the neuronal activity and excitability depression by Mg^{2+} in LRNs agree well with this. Marked suppression of both spontaneous and evoked firing, as well as an increase in threshold potential are presumably due to a decrease in the activation of voltage-gated Na^+ channels through surface charge screening effect of Mg^{2+} (Somjen 2004). Magnesium ions are also found to produce a competitive and reversible blockade of Na^+ channels in central mammalian neurons (Lin et al. 1991, Sang, Meng 2002).

Effect of 20 mmol dm^{-3} Mg^{2+}on input membrane resistance

Finally, in order to further clarify the nature of Mg^{2+} action on the cell membrane of LRNs, we tested the effect of Mg^{2+} on IMR. The resting membrane potential of LRNs in standard Ri was –41.88±1.25 mV (n = 10). Superfusion with 20 mmol dm^{-3} Mg^{2+} Ri caused a highly significant increase of IMR of directly polarized membranes from 13.05±1.84 MΩ in standard Ri to 20.14±2.54 MΩ in Mg^{2+} Ri ($p < 0.01$). Recovery started as soon as Mg^{2+} was washed out. Figure 3 illustrates that superfusion with Mg^{2+} Ri depolarized the cell membrane potential from -34.4 mV by 2.4 mV, reduced the frequency of spontaneous generation of APs from 0.70 Hz to 0.03 Hz, and also increased IMR from 8.42 MΩ to 14.21 MΩ.

While most authors agree that Mg^{2+} depresses neuronal excitability when applied externally, as for the effect of Mg^{2+} on neuronal membrane resistance, the observations differ. A small tendency toward an increase in IMR of cat cortical neurons following micro-iontophoresis of Mg^{2+} was observed by Kato et al. (1968), whereas in frog spinal motoneurons the input resistance was unaffected by Mg^{2+} in the concentration range of 0-11 mmol dm^{-3} (Erulkar et al. 1974). On the contrary, Cameron et al. (2000) and Núñez-Abades et al. (2000) reported that 6 mmol dm^{-3} Mg^{2+} significantly and reversibly increases

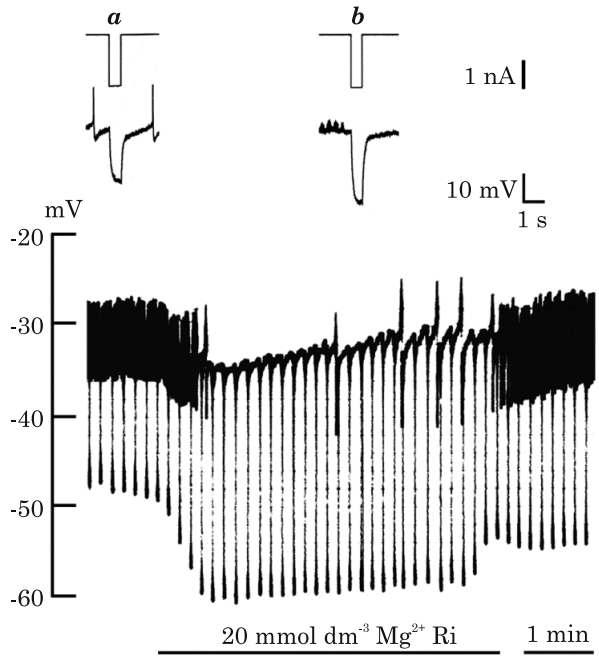

Fig. 3. Effect of 20 mmol dm^{-3} Mg^{2+} on input membrane resistance (IMR) of directly polarized membrane of the leech Retzius neuron. Lower trace is from a mechanical pen recorder. Upper traces represent oscilloscope screen recordings of the delivered hyperpolarizing current pulses and the belonging electrotonic potentials evoked in: *a* – standard Ri solution, and *b* – 20 mmol dm^{-3} Mg^{2+} Ri solution. Application of Mg^{2+} increases IMR

IMR in developing rat brain stem motoneurons. In voltage-clamped rat hippocampal neurons, Dribben et al. (2010) found a decrease in membrane conductance in the presence of 11 mmol dm^{-3} Mg^{2+}. Our finding of Mg^{2+}-induced IMR increase is in good agreement with the latter type of reports.

The responses obtained from LRNs are summarized in Table 1. The observed neuronal inhibition by Mg^{2+} was functionally dominant over its simul-

Table 1

Summary of the effects of bath application of high Mg^{2+} on electrophysiological membrane properties of Retzius neurons in the leech

Item	RMP (mV)	AP frequency (Hz)	AP threshold (mV)	APs evoked	IMR (MΩ)
Control	-42.76±1.24	0.30±0.07	-31.96±1.40	4.29±0.53	13.05±1.84
20 mmol dm^{-3} Mg^{2+}	-38.56±1.19	0.03±0.01	-28.30±2.45	1.57±0.52	20.14±2.54
Δ	4.20±0.49	-0.27±0.07	3.66±1.29	-2.71±0.47	7.09±0.84
n	10	7	5	7	10
p	<0.01	<0.01	<0.05	<0.01	<0.01

Data presented as mean±SEM; RMP – resting membrane potential, AP – action potential, IMR – input membrane resistance, Δ – difference, *n* – number of trials, *p* – level of significance

taneous minor excitatory effect on the membrane potential, although the mixed effects of Mg^{2+} still raise the question of which ones predominate *in vivo*. Suspecting that the inhibitory action of Mg^{2+} on our model may result from the depression of tonic excitatory drive on LRNs, we applied high Mg^{2+} in order to block chemical synaptic transmission, as discussed previously. Under the experimental conditions of diminished synaptic transmission, the nature of the suppression produced by high Mg^{2+} must be intrinsic to the neuron itself and likely to be due to modulatory actions of Mg^{2+} on ion channel function. The blockade of ion channels by Mg^{2+} should produce an increase in IMR, as our results indeed have shown. The Mg^{2+}-induced increase in IMR of LRNs was immediate, significant and sustained all throughout Mg^{2+} exposure. This could confirm the previously proposed mechanism of action through Na^+ channel blockade by Mg^{2+}. The initial effect of transient membrane potential hyperpolarization and diminished tonic firing of LRNs, together with a decrease in membrane excitability and conductivity could all relate to a reduction of the inward Na^+ current by Mg^{2+}, as previously mentioned.

Since Mg^{2+}_e and Mg^{2+}_i ions may act as important physiological regulators of both Na^+ and K^+ transmembrane ion movement (Bara et al. 1993, Guiet-Bara et al. 2007), it is necessary to consider possible Mg^{2+} effects on K^+ channels of LRNs as well. A tonic Ca^{2+}-activated K^+ current ($I_{K(Ca)}$), a major outward current in these cells, is an important regulator of cell membrane excitability and activity pattern in LRNs (Dean, Leake 1988, Stewart et al. 1989, Kleinhaus, Angstadt 1995). Therefore, our findings of a consistent increase in IMR yet with the opposing actions of high Mg^{2+} solution application on the membrane potential of LRNs could all be attributable to several mechanisms of action in a distinct temporal sequence of events. First, a transient membrane hyperpolarizing effect of Mg^{2+} may result from the blockade of leakage Na^+ current by Mg^{2+}_e, as discussed previously. Next, Mg^{2+} influx into the cell due to the activation of electrogenic Na^+/Mg^{2+} antiport system could account for the subsequent Mg^{2+}-induced depolarization. The resulting increase in $[Mg^{2+}]_i$ could, however, differently modulate the function of Ca^{2+}-activated K^+ channels (K_{Ca}), either by potentiating or inhibiting $I_{K(Ca)}$. Mg^{2+}_i is found to potentiate the activation of K_{Ca} by Ca^{2+} in cultured hippocampal neurons (Mclarnon, Sawyer 1993). The potentiation of $I_{K(Ca)}$ by Mg^{2+}_i in LRNs could possibly contribute to the suppression of spontaneous and evoked activity. However, although this role of Mg^{2+} ions as activators of K_{Ca} channels in LRNs cannot be completely ruled out, it did not prove to be the dominant mechanism of Mg^{2+} action on our cell model, since superfusion with Mg^{2+} resulted in an increase rather than a decrease in IMR, while the membrane potential depolarization overcame the initial hyperpolarization. Another and equally plausable interpretation is a contrary mechanism, whereby Mg^{2+}_i inhibits $I_{K(Ca)}$. In cultured rat skeletal muscle cells (Ferguson 1991) and vascular myocytes (Morales et al. 1996) Mg^{2+} is found to reduce the conductance of K_{Ca} channels. The absence of a complete block of both spontaneous and evoked activity with Mg^{2+} we observed on LRNs could even speak in favour of

such a blocking Mg^{2+} effect on K_{Ca} channels. Thereby, Mg^{2+} could have a certain role of a modulator of $I_{K(Ca)}$ in the regulation of excitability of LRN cell membrane. However, further studies are necessary to enlighten the matter.

CONCLUSIONS

1. In the present study, an overall inhibitory influence of high extracellular Mg^{2+} on membrane properties and electrical activity of LRNs was found. The neurodepressive effects of Mg^{2+} consisted of a strong and almost complete suppression of spontaneous activity, an increase in AP threshold, a decrease in cell excitability, and an increase in IMR.

2. In all aspects, the blocking action of Mg^{2+} was rapid in onset, significant and reversible. It was accompanied by a minor membrane depolarization that may be due to Mg^{2+} influx into the cell. However, our results clearly suggest that the predominant Mg^{2+} action in LRNs is inhibitory.

3. A probable underlying mechanism common to all suppressive Mg^{2+} effects is discussed to be the blockade of Na^{+} channels by Mg^{2+}, but multiple Mg^{2+} effects on different ion channels are possible, including the modulation of K_{Ca} channel activity.

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