Effects of hypomagnesemia on transmitter actions in neocortical slices

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- 1 The effects of hypomagnesemia on the neuronal responses induced by iontophoretically applied acetylcholine, glutamate, N-methylaspartate (NMDA) and γ -aminobutyric acid (GABA) were investigated using intracellular recording techniques in *in vitro* slices of sensorimotor cortex (guinea-pigs).
- 2 Perfusion with Mg-free media, with or without tetrodotoxin (TTX), induced a small hyperpolarization (~ 4 mV) and a small decrease ($\sim 10\%$) in the input resistance of neurones. During TTX-blockade of Na-spike genesis, spontaneous depolarizing waves of low frequencies were observed in neurones of slices under Mg-free conditions.
- 3 The effects of acetylcholine and to a lesser extent, GABA actions, were depressed in a dose-dependent, reversible manner by decreases in the $[\text{Mg}^{2+}]$ of the perfusing media. In neurones of slices that had been incubated in Mg-free artificial cerebrospinal fluid to ensure a maximal depletion, the responses to these transmitters were potentiated by each sequentially administered increase in extracellular $[\text{Mg}^{2+}]$. The actions of NMDA were potentiated during perfusion of Mg-free media. However, the responses to glutamate, which may activate receptors for NMDA, were either depressed or unchanged under these conditions.
- 4 A regulatory role for external Mg cations in the responses of neocortical neurones to the transmitter substances, acetylcholine and GABA, can be inferred from these investigations which simulate hypomagnesemia. The dose-dependent depression of GABA actions by low extracellular $[\text{Mg}^{2+}]$ additionally provides a plausible mechanism that may contribute to the neuronal hyperexcitability that is observed during conditions of hypomagnesemia.

Introduction

In man and experimental animals, hypomagnesemia is associated with manifestations of central nervous system (CNS) dysfunction (Martindale & Heaton, 1964; Agus *et al.*, 1982). These can be attributed to the following mechanisms, based on *in vitro* investigations of the extracellular effects of Mg on CNS neurones: (1) removal of the well-known antagonistic actions of Mg on Ca-entry at both pre- and postsynaptic sites (Katz & Miledi, 1969; Lambert & Heinemann, 1986; Czèh & Somjen, 1989), (2) reduced surface charge screening on neuronal membranes and an associated facilitation of inward ionic currents and action potentials (Frankenhauser & Hodgkin, 1957; Llinás & Walton, 1980), and (3) removal of a voltage-dependent Mg-blockade of excitatory amino acid actions at N-methyl-D-aspartate (NMDA)-receptors (Thomson, 1986; Stanton *et al.*, 1987; MacDonald *et al.*, 1987; Mayer *et al.*, 1988; Collingridge & Lester, 1989). Another obvious possibility is that the hypomagnesemia may interfere with the role of Mg in regulating a wide variety of enzymatic reactions and phosphorylation-dependent processes, including second messenger systems (Gurwitz & Sokolovsky, 1980; Grubbs & Maguire, 1987; Stelzer *et al.*, 1988), that are essential for neuronal excitability in the CNS. An explanation for this effect may require that the decrease in extracellular $[\text{Mg}^{2+}]$ ($[\text{Mg}^{2+}]_o$) would result in a corresponding drop in intracellular $[\text{Mg}^{2+}]$ (Baker & Crawford, 1972; Heinonen & Akerman, 1986) which would compromise certain receptor sensitivities to transmitter actions.

Here, we investigated the possibility that hypomagnesemia in *in vitro* slice preparations may modify neuronal responses to known transmitter substances such as acetylcholine, glutamate and γ -aminobutyrate (GABA) in neocortical neurones (Krnjević, 1974). Some of these results have been published in preliminary form (El-Beheiry & Puil, 1989a).

Methods

Slice preparations and recording

Sensorimotor cortex was identified in halothane-anaesthetized guinea-pigs, excised, and cut into ~ 500 μm thick slices

according to previously described procedures (El-Beheiry & Puil, 1989b). The slices were submerged in artificial cerebrospinal fluid (ACSF) oxygenated with a 95% O_2 /5% CO_2 mixture and kept at room temperature until required for recording (bath temperature, 32–34°C). The constituents of the ACSF were (in mM): NaCl 124, KCl 3.75, KH_2PO_4 1.25, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 2, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 2, dextrose 10 and NaHCO_3 26. When changes were made in the $[\text{Mg}^{2+}]_o$ by altering the ACSF composition, the total concentration of divalent cations was maintained constant by appropriately increasing or decreasing the $[\text{Ca}^{2+}]$. In 4 experiments, Ca-deficient ACSF was made by equimolar substitution of CoCl_2 for CaCl_2 . The techniques for intracellular recording from neocortical neurones in *in vitro* slice preparations with K_2SO_4 (0.6 M) filled electrodes (cf. Eccles, 1964) have been described; these descriptions included measurements of input resistance by intracellular injections of hyperpolarizing current pulses (100–150 ms duration) and current-voltage relationships (El-Beheiry & Puil, 1989b). In cases where a change in resting membrane potential was observed during an imposed alteration in $[\text{Mg}^{2+}]_o$, d.c.-injection was used to return the resting potential to its initial level.

Drugs and their applications

Acetylcholine (0.5 M, pH 4.5), glutamate (0.5 M, pH 8.5), N-methyl-D-aspartate (NMDA; 100 mM, pH 9) and γ -aminobutyrate (GABA; 100 mM, pH 4.5) (all obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.), or NaCl for control applications, were administered by iontophoresis from a three- or five-barrelled microelectrode which was inserted into the dendritic region at a distance of ~ 50 μm from the location of neuronal impalement. The substances were applied consistently at recurring intervals (10–60 s) for observations of their interactions with imposed changes in $[\text{Mg}^{2+}]_o$. The durations of the applications were 4 to 7 s for GABA, 6 s for glutamate and 7 to 20 s for acetylcholine. In 20 neurones, tetrodotoxin (TTX; 1–1.5 μM) (Sigma Chemical Co.) was applied in the ACSF to block action potential-dependent transmitter release.

Results

Successful penetrations of neurones were obtained mostly in regions corresponding to layers IV–V of typical cortex. The effects of a reduction in the $[Mg^{2+}]_o$ were investigated in 32 sensorimotor neurones.

Membrane properties and spikes

Before hypomagnesemia, the mean resting membrane potential and mean input resistance were -70.4 ± 7.3 (mean $\pm$ s.d.) mV and $48.8 \pm 18.5 M\Omega$, respectively ($n = 24$). Perfusion of the slices with Mg-free media, with or without the application of TTX, induced a slight hyperpolarization (3–5 mV) in 8 neurones (cf. Table 1). After 30–45 min in Mg-free and TTX perfusion, the resting membrane potentials in 4 neurones exhibited abruptly appearing episodes of depolarizing oscillations (4–7 mV), each lasting 10–20 s. The input resistances were unchanged in 11 of the neurones, and decreased by $\sim 10\%$ in 5 neurones during the hyperpolarization. The current-voltage relationships in the hyperpolarizing direction displayed an inward rectification in $\sim 85\%$ of the neurones ($n = 24$) which was not significantly affected by the Mg-depletion. Intracellular injections of depolarizing current pulses (~ 100 ms duration) evoked spikes of > 80 mV amplitude and < 2 ms duration, with overshoots of ~ 20 mV. The spikes also were not apparently affected by the Mg-deficient conditions.

Responses to transmitter substances

Applications ($\sim ED_{50}$ s) of acetylcholine, glutamate and GABA produced mean peak depolarizations of 15.8 ± 4.6 , 20.5 ± 7.7 and 21.5 ± 7.8 mV respectively in the 24 neurones. The input resistances of these neurones were either increased ($\sim 15\%$) or unaffected near the peak of the depolarizing responses induced by acetylcholine. In contrast, the input resistances were decreased (35–80%) by glutamate or GABA applications. These responses were obtained either in the absence or presence of TTX ($1.5 \mu M$).

Perfusion of the Mg-free ACSF was commenced shortly after (3–5 min) 3 or more applications of each transmitter substance had produced responses of relatively constant amplitudes. Application of acetylcholine or GABA induced depolarizations which were sharply reduced during Mg-free perfusion (Figure 1a; Table 1). The responses to glutamate were not consistently affected during perfusion with Mg-free media ($n = 9$). The amplitudes of the depolarizations evoked by glutamate application were either depressed by $< 20\%$, unchanged, or enhanced by $\leq 20\%$. Depolarizing responses to NMDA application that were comparable in amplitude to those elicited by glutamate could not be obtained during the perfusion of ACSF containing 2 mM Mg^{2+} ($n = 10$). However, during 5–10 min perfusion of Mg-free media, the application of NMDA evoked a depolarization of ~ 20 mV amplitude in all 10 neurones. All of the above effects of Mg-free media were fully reversible; generally, recoveries were observed at 12–18 min after reperfusion with Mg (perfusion flow rates, 1–3 ml min $^{-1}$).

Figure 1 (a) Blockade of acetylcholine (ACh, 90 nA for 8 s) and GABA (50 nA) actions under conditions of Mg-free perfusion for 10 min. ACh and GABA responses were completely attenuated in a neurone ($V_m = -75$ mV) whereas the effects of glutamate (Glu) application (90 nA) were still evident, although reduced. The hyperpolarization (~ 4 mV) induced by Mg-free perfusion was compensated by d.c.-injection. Complete recovery of potential and transmitter responses was observed at ~ 15 min of reperfusion with Mg. Vertical bar, 15 mV. Time calibration, 8 s (ACh application bar). (b) Dose-response relationships obtained from 18 neurones indicate dependencies of the depolarizations evoked by ACh ($\circ$), GABA ($\diamond$) and Glu ($\triangle$) on $[Mg^{2+}]_o$. Each of the points represents the mean data pooled from at least 4 neurones and vertical lines show s.e.mean.

Dose-response relationships

The depolarizations evoked by acetylcholine, glutamate and GABA were averaged in 18 neurones bathed in conditions containing different concentrations of Mg^{2+} (Figure 1b). The responses to acetylcholine and GABA approached their respective maxima sooner and were larger in peak amplitude, with increasing $[Mg^{2+}]_o$ (not shown). However, changes in the rate of rise and the magnitude of the glutamate responses were very inconsistent and seemed to be largely independent of the $[Mg^{2+}]_o$.

Table 1 Effects of Mg-free media on transmitter responses in sensorimotor neurones

Resting membrane properties	Decrease						
	Hyperpolarization	in input resistance	Depression* of ACh response	Depression of GABA response	Potential of NMDA response	Depression	Glutamate response No change Potentiation**
	8/16	5/16	7/7	13/13	10/10	3/9	4/9 2/9

* Defined as $> 20\%$ attenuation of control amplitudes of ACh-induced depolarization.

** Defined as $> 20\%$ increase of control amplitudes of glutamate-induced depolarization.

Figure 2 Gradual potentiation of the effects of acetylcholine (ACh) application (85 nA) in a neurone ($V_m = -70$ mV) that corresponded to increases in $[Mg^{2+}]_o$. The slice had been previously incubated in Mg-free media for ~2 h. Glutamate (Glu) was applied with a 100 nA current for 5 s. The responses to ACh and Glu were obtained at ~7 min of perfusion at each concentration of Mg^{2+} . Vertical bar, 15 mV. Time calibration, 9 s (ACh application bar).

Effects of sequentially increasing $[Mg^{2+}]_o$

In a separate series of experiments, the neuronal responses to the transmitter substances were investigated under conditions where the slices had been first incubated for 3–7 h in Mg-free ACSF to ensure a maximal depletion of $[Mg^{2+}]_o$ (cf. Sutor *et al.*, 1987). Neurones ($n = 8$) in slices that had been incubated in Mg-free ACSF since cortical excision showed pronounced spontaneous (presumably synaptic) activities. During perfusion of increasing levels of $[Mg^{2+}]_o$ the spontaneous firing gradually subsided, although synaptic transients and spiking were still evident at the highest concentration (cf. Figures 2 and 3). This activity was associated with a small depolarization (~4 mV), although input resistance remained unchanged from that observed in Mg-free conditions in 6 out of the 8 neurones.

Under the Mg-free conditions none of the neurones responded to the applications of acetylcholine. However, during perfusion with ACSF containing 0.5 mM Mg, depolarizations could be evoked with acetylcholine. These increased in amplitude with perfusion of ACSF containing 1.0 and 2.0 mM $[Mg]$ (5 out of 8 neurones; Figure 2). In contrast to the absence of acetylcholine sensitivity, the neurones responded to GABA and glutamate applications during Mg-free conditions. However, the GABA-induced depolarizations were potentiated in a dose-dependent manner by the administration of additional Mg^{2+} (Figure 3). During Mg-free perfusion, application of glutamate depolarized all 8 neurones and a build-up in $[Mg^{2+}]_o$ to 0.5, 1.0 and 2.0 mM did not consistently produce much change in these responses.

In 2 of the above neurones, the responses to acetylcholine were entirely blocked, and the GABA responses were depressed, when the slices were perfused with Mg-free ACSF, demonstrating a reproducibility in the reversible effects of hypomagnesemia. In general, the results obtained from neurones under Mg-free conditions after cortical excision, and the effects of the cumulative applications of Mg, were entirely consistent with the results obtained from neurones under conditions where the $[Mg^{2+}]_o$ had been 'acutely' lowered by perfusion of Mg-free media.

Figure 3 Continuous record shows that γ -aminobutyric acid (GABA) application (80 nA) produced responses which were gradually potentiated by sequential increases in $[Mg^{2+}]_o$. The slice had been previously incubated in Mg-free artificial cerebrospinal fluid for ~3 h. ($V_m = -72$ mV).

Effects of changes in divalent cations in the external media on the transmitter responses

In order to investigate the possibility that the increase in the external $[Ca^{2+}]$ used to maintain the total cationic concentration constant contributed to the observed effects, slices were perfused with ACSF solutions containing 4 mM Ca^{2+} . No significant changes were observed in the depolarizations evoked by acetylcholine, glutamate and GABA under these Ca-rich conditions. Two other neurones were bathed in Mg-free ACSF which contained only 2 mM Ca^{2+} . In this medium, the depressions of the acetylcholine and GABA responses were very similar in amplitude and duration to those obtained in neurones bathed in hypomagnesian conditions, i.e., with 4 mM Ca^{2+} .

Because of the variability in the effects of a reduction or a build-up in $[Mg^{2+}]_o$ on the responses to glutamate, we also investigated the effects when systematic changes were made in the divalent cation concentration of the perfusing media. Most of these experiments were carried out without changing the total cationic concentration and during concomitant application of TTX. Exclusion of both Ca and Mg from the perfusing media together with their substitution by 4 mM Co^{2+} , attenuated the peak depolarization produced by glutamate application by $38.8 \pm 7.5\%$ in 3 out of the 4 neurones (Figure 4a). Substitution of Ca alone by Co^{2+} in the external media suppressed the glutamate responses by $15 \pm 5.7\%$ in 4 neurones (Figure 4b). Recovery was complete after 15–20 min at a perfusion flow rate of 2–3 ml min⁻¹.

Discussion

In the present investigations, hypomagnesemia was produced in *in vitro* slice preparations by substituting Ca for Mg in the perfusing media, such that the total divalent cation concentration was constant at 4 mM. Any interference with the surface charge screening of the membrane that would have facilitated an activation of inward currents was thereby avoided (Frankenhauser & Hodgkin, 1957; Llinás & Walton, 1980). The effects induced by the simulated hypomagnesemia could not be attributed to changes in the extracellular $[Ca^{2+}]$ (cf. Results) and one can be reasonably confident that they are mostly due to changes in the extracellular concentration of Mg (Frankenhauser & Hodgkin, 1957; McLaughlin *et al.*, 1971).

Unlike hippocampal neurones (Mody *et al.*, 1987), an exposure to Mg-free media hyperpolarized neocortical neurones and decreased their input resistances. Conversely, a depolar-

Figure 4 Effects of changing the external $[Ca^{2+}]$ and $[Mg^{2+}]$ on responses to glutamate applications (100 nA) in two neurones ($V_m = -68$ mV in a, -78 mV in b). Responses to glutamate (Glu) were depressed in Ca- and Mg-free artificial cerebrospinal fluid (ACSF) (a) more than in the absence of Ca alone (b). Recovery was observed at ~ 15 min in (a), and at ~ 20 min in (b), after reperfusion with control ACSF.

ization with a magnitude comparable to the peak hyperpolarization was observed on several occasions during the buildup of $[Mg^{2+}]_o$ from hypomagnesemia. The hyperpolarization during hypomagnesemia may be attributed to an interruption of tonic, blocking actions of Mg on resting Ca-activated K-channels, (cf. Llinás & Walton, 1980; Begenisich, 1988). Such blocking actions have been observed in spinal motoneurons where intracellular injections of Mg^{2+} produce membrane depolarization by reducing a Ca-activated K-conductance (Krnjević *et al.*, 1976). To explain the neocortical results (see below) this mechanism would probably require a dependence of the internal $[Mg^{+}]$ on $[Mg^{2+}]_o$, which has been suggested to be a general feature of Mg regulation in nerve cells (Heinonen & Akerman, 1986; Baker & Crawford, 1972).

Suppression of the acetylcholine-induced responses

A dose-dependent suppression of acetylcholine-induced depolarizations in neurones by removal of Mg from the extracellular medium has not been described previously. External applications of Mg by iontophoresis have been found to depress acetylcholine- and carbachol-evoked spike firing in the spinal cord and dorsal root ganglion (Davies & Watkins, 1977), but not to antagonize nicotinic actions on cerebellar neurones (Garza *et al.*, 1987). Although the K-conductance mechanism is different, a blockade of the muscarinic actions by a reduced $[Mg^{2+}]_o$ has been observed in cardiac muscle (Kurachi *et al.*, 1986). In the neocortex, the interactions of acetylcholine with muscarinic receptors depolarize pyramidal neurones by reducing their membrane conductances for K (Krnjević *et al.*, 1971). In sympathetic ganglion neurones the muscarinic actions involve a reduction in the Ca-activation of K-channels (Belluzzi *et al.*, 1985; Wanke *et al.*, 1987).

The depression of muscarinic responses in neocortical neurones can be attributed to one or more of the following mechanisms: (1) a decrease in $[Mg^{2+}]_o$ may remove the tonic inhibition of a Ca-conductance, thereby increasing Ca-dependent K-efflux (cf. Belluzzi *et al.*, 1985; Wanke *et al.*, 1987); this would effectively counteract the depolarizing effects of the acetylcholine. (2) Decreasing $[Mg^{2+}]_o$ may cause corresponding decrements in intraneuronal $[Mg^{2+}]$ (Heinonen & Akerman, 1986; Baker & Crawford, 1972) that would change the internal Ca^{2+}/Mg^{2+} ratio; this could interfere with the actions of acetylcholine (Krnjević *et al.*, 1976). (3) A decrease in internal $[Mg^{2+}]$ might also lead to a reduction in gua-

nosine 5'-triphosphate (GTP) binding to the agonist/receptor complex, thereby producing the depression of acetylcholine responses (Gurwitz & Sokolovsky, 1980; Aronstam *et al.*, 1985). (4) Low $[Mg^{2+}]_o$ favours a conversion of the muscarinic receptor with high affinity binding sites to low affinity conformational states in the cerebral cortex, whenever Mg is added to the incubating medium (Birdsall *et al.*, 1984); this is consistent with the observed reduction in the responses to acetylcholine.

$[Mg^{2+}]_o$ does not significantly affect glutamate-induced responses

As in previous intracellular investigations on rat and human neocortical slices (Thomson *et al.*, 1985; Sutor *et al.*, 1987; Avoli *et al.*, 1987), no substantial changes in the neuronal responses evoked by glutamate were observed in guinea-pig neocortical slices under Mg-free conditions. In the frog and rat spinal cords, large reductions or increases in $[Mg^{2+}]_o$ do not produce appreciable effects on the extracellularly recorded responses to glutamate (Ault *et al.*, 1980; cf. Lacey & Nistri, 1988). The absence of effects in neocortex could be due to a failure of Mg^{2+} to influence significantly the binding of glutamate to its receptors (Baudry & Lynch, 1979), or may result from the interactions of glutamate predominantly with non-NMDA-sensitive sites in layers IV and V (Monaghan & Cotman, 1985). The observed trend towards a depression of the glutamate responses in the absence of external Ca (cf. Figures 1 and 4) may be significant in view of the possible charge contribution of Mg^{2+} to the depolarization evoked by glutamate actions (Pumain *et al.*, 1988).

Attenuation of GABA-depolarizations by Mg-free ACSF

The effects of $[Mg^{2+}]_o$ on the activation of Cl-currents by GABA-receptor interactions have not been observed previously in mammalian CNS neurones. However, extracellular applications of Zn^{2+} , Co^{2+} , Ni^{2+} or Cd^{2+} antagonize the responses to GABA in various vertebrate neurones (Kaneko & Tachibana, 1986). Intracellular Mg^{2+} has been suggested to be essential for preventing a 'rundown' of GABA_A-receptor function (Stelzer *et al.*, 1988; Gyenes *et al.*, 1988). Without clear biochemical evidence that the $[Mg^{2+}]_o$ affects the binding characteristics of GABA_A-receptors, one may assume that the Mg-dose-dependent inhibition of the depolarizations produced by GABA applications in our investigations is due to a decrease in the intracellular $[Mg^{2+}]$ subsequent to the removal of $[Mg^{2+}]_o$ (Baker & Crawford, 1972; Heinonen & Akerman, 1986); this could lead to a 'rundown' of GABA_A receptor function or to a reduced affinity of the receptors for GABA (cf. Inoue *et al.*, 1986).

Enhancement of neuronal excitability in Mg-free ACSF

A reduction of $[Mg^{2+}]_o$ is known to enhance neuronal excitability in cortical systems including the hippocampus and entorhinal (Walter *et al.*, 1986; Stanton *et al.*, 1987; Mody *et al.*, 1987; Hamon *et al.*, 1987) as well as sensorimotor cortex (Thomson, 1986). Several mechanisms have been proposed for the presumed regulating role of $[Mg^{2+}]_o$ (cf. Introduction). The dose-dependent depression of the responses to GABA, a cortical inhibitory transmitter (Krnjević, 1974), which was observed in the present investigations, provides an additional mechanism that may contribute to the neuronal hyperexcitability observed during conditions of hypomagnesemia.

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