Sodium–magnesium antiport in Retzius neurones of the leech *Hirudo medicinalis*

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1. Intracellular free magnesium ($[Mg^{2+}]_i$) and sodium ($[Na^+]_i$) concentrations were measured in Retzius neurones of the leech *Hirudo medicinalis* using ion-sensitive microelectrodes.

2. The mean steady-state values for $[Mg^{2+}]_i$ and $[Na^+]_i$ were 0·46 mM (pMg, 3·34 ± 0·23; range, 0·1–1·2 mM; $n = 32$) and 8·95 mM (pNa, 2·05 ± 0·15; range, 5·1–15·5 mM, $n = 21$), respectively, at a mean membrane potential (E_m) of −35·6 ± 6·1 mV ($n = 32$). Thus, $[Mg^{2+}]_i$ is far below the value calculated for a passive distribution (16·9 mM) but close to the equilibrium value calculated for a hypothetical 1 Na^+–1 Mg^{2+} antiport (0·41 mM).

3. Simultaneous measurements of $[Mg^{2+}]_i$, $[Na^+]_i$ and E_m in Retzius neurones showed that an increase in the extracellular Mg^{2+} concentration ($[Mg^{2+}]_o$) resulted in an increase in $[Mg^{2+}]_i$, a parallel decrease in $[Na^+]_i$ and a membrane depolarization, while a decrease in $[Mg^{2+}]_o$ had opposite effects. These results are compatible with calculations based on a 1 Na^+–1 Mg^{2+} antiport.

4. Na^+ efflux at high $[Mg^{2+}]_o$ still occurred when the Na^+–K^+ pump was inhibited by the application of ouabain or in K^+-free solutions. This efflux was blocked by amiloride.

5. In the absence of extracellular Na^+ ($[Na^+]_o$), no Mg^{2+} influx occurred. Mg^{2+} influx at high $[Mg^{2+}]_o$ was even lower than in the presence of $[Na^+]_o$. Mg^{2+} efflux was blocked in the absence of $[Na^+]_o$.

6. The rate of Mg^{2+} extrusion was reduced by lowering $[Na^+]_o$, even if the Na^+ gradient across the membrane remained almost unchanged.

7. Mg^{2+} efflux was blocked by amiloride (half-maximal effect at 0·25 mM amiloride; Hill coefficient, 1·3) but not by 5-(*N*-ethyl-*N*-isopropyl)-amiloride (EIPA).

8. No changes in intracellular Ca^{2+} and pH (pH_i) could be detected when $[Mg^{2+}]_o$ was varied between 1 and 30 mM.

9. Changing pH_i by up to 0·4 pH units had no effect on $[Mg^{2+}]_i$.

10. The results suggest the presence of an electrogenic 1 Na^+–1 Mg^{2+} antiport in leech Retzius neurones. This antiport can be reversed and is inhibited by low extracellular and/or intracellular Na^+ and by amiloride.

The free Mg^{2+} concentration ($[Mg^{2+}]_i$) in single cells is between one and three orders of magnitude lower than what would be expected from a passive distribution across the cell membrane and varies between 0·1 and about 1 mM, depending on the preparation (for review see Maguire, 1990; McGuigan, Blatter & Buri, 1991). Thus, mechanisms must exist which actively extrude Mg^{2+} from the cytoplasm. Several types of transport mechanisms have been postulated: ATP-independent, reversible (Flatman & Smith, 1990) and ATP-dependent, non-reversible (Lüdi & Schatzmann, 1987; Frenkel, Graziani & Schatzmann, 1989; Buri & McGuigan, 1990; see, however, Buri *et al.* 1993) 1 Na^+–1 Mg^{2+} antiport, ATP-dependent 2 Na^+–1 Mg^{2+} antiport (Günther, Vormann, Cragoe & Höllriegel, 1989; Günther, Vormann & Höllriegel, 1990); ATP-dependent 3 Na^+–1 Mg^{2+} antiport (Féray & Garay, 1988); Na^+, K^+–Mg^{2+} antiport (Rasgado-Flores & Gonzales-Serratos, 1990); K^+-dependent Mg^{2+} extrusion (Montes, Sjodin, Wu, Chen, Yergey & Vieira, 1990) and Mg^{2+} and Cl^- extrusion (Günther *et al.* 1989).

In many cell types Mg^{2+} extrusion is sensitive to amiloride, quinidine and imipramine (Féray & Garay, 1986, 1988; Günther *et al.* 1989, 1990; Flatman & Smith, 1990). However, Mg^{2+} extrusion is insensitive to vanadate, ouabain, bumetanide and stilbene disulphonic acids, the inhibitors of the E_1E_2-type cation pumps (Na^+–K^+-ATPase,

Ca^{2+}-ATPase), Na^+–K^+-ATPase, Na^+–K^+–Cl^- cotransport and anion transport, respectively, (for review see Beyenbach, 1990).

ATP-independent Na^+–Mg^{2+} antiport should be reversible, i.e. Mg^{2+} should be driven into the cell if Na^+ is removed from the extracellular medium. However, such a reversal frequently cannot be induced experimentally (Lüdi & Schatzmann, 1987; Frenkel *et al.* 1989). Therefore, ATP dependence has been proposed for a number of Na^+–Mg^{2+} antiporting systems, either in the form of an activation by phosphorylation of the carrier protein (DiPolo & Beaugé, 1988; Günther *et al.* 1990) or by a direct coupling of the Na^+ and Mg^{2+} transport to ATP splitting (Frenkel *et al.* 1989). In the latter case it is difficult to understand why such an ATP-driven Mg^{2+} pump should move Na^+ into the cell, thereby weakening the Na^+ gradient (Flatman & Smith, 1990). Calculations of the electrochemical potentials of Na^+ and Mg^{2+} demonstrate that even a 1 Na^+–1 Mg^{2+} antiport is able to maintain $[Mg^{2+}]_i$ at physiological levels (Flatman & Smith, 1990; present results). Hence, from an energetic point of view there should be no need for a direct coupling of a Na^+–Mg^{2+} antiport to ATP splitting.

In the present study the regulation of intracellular Mg^{2+} was investigated in the Retzius neurones of the leech *Hirudo medicinalis*. This preparation provides several advantages for the application of ion-sensitive microelectrodes: (1) segmental ganglia of the leech contain a constant, low (~400) number of neurones. The neurones can be identified individually by their position within the ganglion, their size and their electrophysiological characteristics: (2) with a diameter of ~80 μm, Retzius neurones are large enough to tolerate impalement by two microelectrodes to monitor up to three parameters ($[Mg^{2+}]_i$, $[Na^+]_i$ or pH_i and the membrane potential (E_m)) simultaneously for several hours: (3) since resting levels of $[Ca^{2+}]_i$ and $[K^+]_i$ in Retzius neurones are well known (Deitmer & Schlue, 1981; Deitmer, Eckert & Schlue, 1987), and control measurements for these ions can easily be carried out, the interference of Ca^{2+} and K^+ ions on the $[Mg^{2+}]_i$ and $[Na^+]_i$ sensors could be minimized in the present experiments.

Our results support the existence of a reversible, ATP-independent, electrogenic 1 Na^+–1 Mg^{2+} antiport which is inhibited by amiloride and by a low extracellular and/or intracellular Na^+ concentration.

Some of the results presented here have been published in abstract form (Günzel & Schlue, 1994).

METHODS

Preparation

All experiments were carried out on Retzius neurones located in segmental ganglia of the leech *Hirudo medicinalis*. Segmental ganglia were dissected with the ganglion capsule intact as described by Deitmer & Schlue (1981). Single ganglia were transferred to the experimental chamber and fixed ventral side up by piercing the connectives with insect pins. The preparation was continuously superfused with saline at a rate of 20 chamber volumes per minute (5 ml min^{-1}) at room temperature (20–25 °C).

Bath solutions

Standard leech saline (SLS) contained (mM): NaCl, 85; KCl, 4; $CaCl_2$, 2; $MgCl_2$, 1; Hepes, 10; pH 7·4 adjusted with NaOH. In experiments where the Na^+ or the K^+ content of the saline was reduced, these ions were substituted by equimolar amounts of *N*-methyl-D-glucamine$^+$ ($NMDG^+$). $NMDG^+$ was chosen as a substituent, as, in agreement with Buri *et al.* (1993), we never found any interference of $NMDG^+$ at the Mg^{2+}-sensitive microelectrode. When the Mg^{2+} content of the saline was increased, an appropriate amount of NaCl or NMDG-Cl was replaced by $MgCl_2$ to maintain osmolarity. Control experiments were carried out in solutions with an increased Mg^{2+} content in which the excess Cl^- was substituted either by gluconate or by SO_4^{2-}. During pH_i measurements intracellular pH changes were induced by exposing the neurones to CO_2–HCO_3^--buffered solutions. In these solutions 22 mM NaCl was exchanged with an equal amount of $NaHCO_3$. The solutions were equilibrated with 5% CO_2–95% O_2.

Microelectrodes

Microelectrodes were pulled from borosilicate glass capillaries (single-barrelled: Clark GC150F-15; double-barrelled: 'theta', Clark TGC200-15; 'twisted', Clark GC150F-15 and Clark GC100F-15; triple-barrelled: Clark TGC200-15 and Clark GC150F-15, Clark, Reading, UK) with a vertical puller (Narashige, Tokyo, Japan).

Single-barrelled microelectrodes were used as internal reference electrodes. Their electrical resistance when filled with 3 M KCl was 20–30 MΩ.

Both barrels of the theta electrodes were silanized by exposing their back ends to the vapour of hexamethyldisilazane (Fluka, Buchs, Switzerland) at 40 °C for 45–60 min and subsequently baking them at 200 °C for at least 2 h. Silanized electrodes could be stored on silica gel for several weeks. One day prior to the use of the electrode, the tip of one barrel was filled with the Mg^{2+} sensor ETH 5214 (Mg^{2+} ionophore II, cocktail A, Fluka), that of the other barrel was filled with the Na^+ sensor ETH 227 (Na^+ ionophore I, cocktail A, Fluka). Air bubbles within the sensor columns were removed by exposing the electrodes to low pressure in a desiccator for ~15 min. Then the Mg^{2+} barrel was backfilled with the 1 mM Mg^{2+} calibration solution (for composition see below) or 100 mM $MgCl_2$, the Na^+ barrel was filled with 100 mM NaCl. The electrodes had to be stored with their tips in standard leech saline for at least 12 h to obtain stable responses.

To make 'twisted' or triple-barrelled electrodes, two glass capillaries with differing diameters ('twisted', 1·5 and 1 mm; triple-barrelled, 2 mm theta and 1·5 mm) were glued together. The glass was softened in the heating coil of the microelectrode puller and the thinner capillary twisted 180 deg around the thicker one. The glass was then allowed to cool down before both capillaries were pulled out together to obtain one single tip. The thicker barrel of such an electrode was silanized as described above, while the thinner barrel was prevented from being silanized by filling it with distilled water. The water evaporated during the baking of the electrode, so that the barrel could then be filled with 3 M KCl to be used as a reference barrel.

For the 'twisted' electrodes, the silanized barrel was filled with the pH sensor ETH 1907 (hydrogen ionophore II, cocktail A, Fluka) and the pH 7·67 calibration solution (composition see below) as described for the theta electrodes. For triple-barrelled electrodes, the two silanized channels of the theta capillary could be filled with any combination of two of the three ion sensors.

Filled electrodes were stored with their tips immersed in standard leech saline. They could be used for up to 3 days after being filled. During the experiments, these electrodes showed a drift of $-1{\cdot}6 \pm 6{\cdot}5$ mV h^{-1}, $n = 117$ (Mg^{2+}-sensitive channel); $-0{\cdot}6 \pm 6{\cdot}3$ mV h^{-1}, $n = 94$ (Na^+-sensitive channel); $1{\cdot}6 \pm 8{\cdot}2$ mV h^{-1}, $n = 31$ (pH-sensitive channel) and $-2{\cdot}8 \pm 5{\cdot}0$ mV h^{-1}, $n = 121$ (reference channel). For correction, the drift was assumed to be linear throughout the experiment.

Calibration procedure

Both the Mg^{2+} sensor (ETH 5214) and the Na^+ sensor (ETH 227) are prone to the influence of interfering ions, mainly through K^+ at the Mg^{2+} sensor and through Ca^{2+} at the Na^+ sensor. All calibration solutions, therefore, contained an ionic background which simulated intracellular conditions. Care, however, had to be taken that during the experiments large alterations in $[Ca^{2+}]_i$ and $[K^+]_i$ were avoided.

As Mg^{2+} and Na^+ electrodes did not behave linearly in the physiological range, they were each calibrated with a series of seven calibration solutions (Fig. 1). The resulting calibration curves were fitted with the Nicolsky–Eisenman equation (Ammann, 1986), using the spreadsheet 'Quattro Pro 5.0' (Borland, Langen, Germany). Electrodes were only used if their slope in the linear range of the electrode was at least 90% of the Nernstian slope and if their detection limit was below the values recorded during an experiment (about 75% of the Na^+–Mg^{2+} theta electrodes). pH electrodes were calibrated only at two different pH values, as the pH sensor used behaves linearly over a wide pH range. Electrodes were used in the experiments when the electrode slope was > -52 mV (pH unit)$^{-1}$.

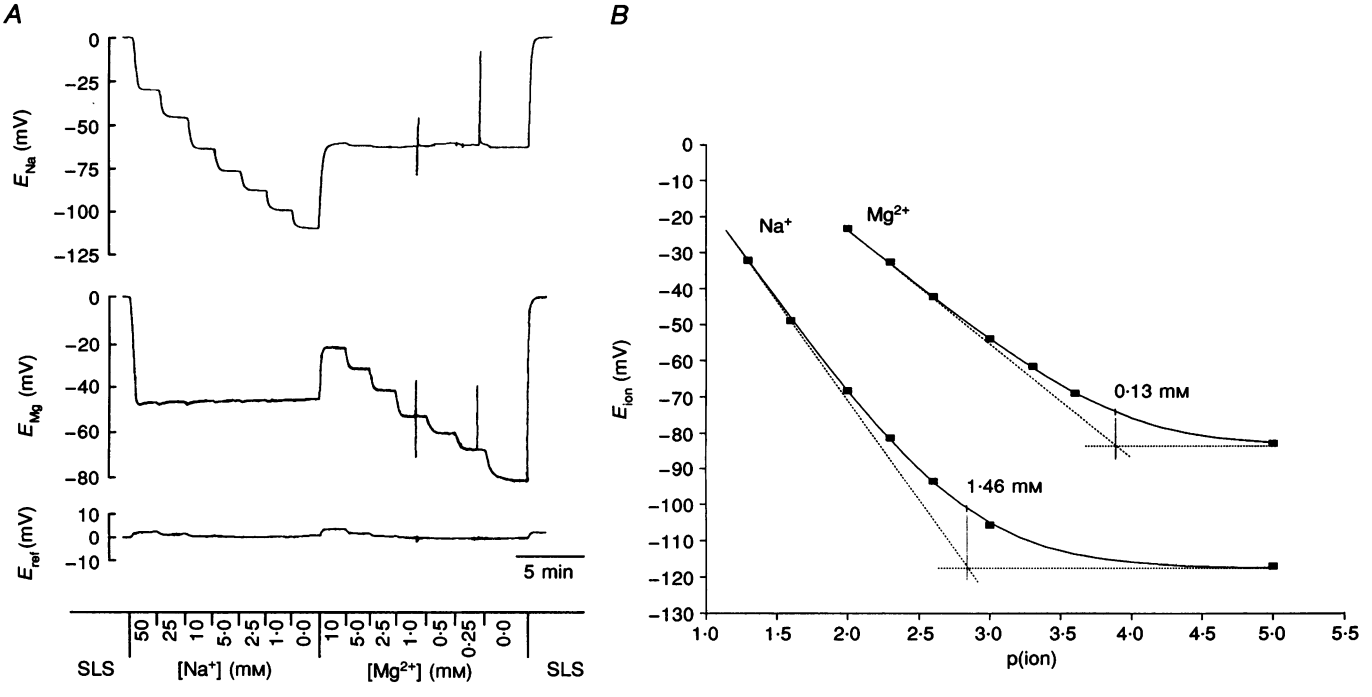

Figure 1. Calibration of a double-barrelled Na^+- and Mg^{2+}-sensitive microelectrode

The single ion potentials (E_{Na}, E_{Mg}) and the reference potential (E_{ref}) were recorded during calibration (*A*). E_{Na} and E_{Mg} were grouped as E_{ion} and plotted against the negative logarithm of the ion concentration (p(ion)) (*B*). The continuous line reflects values calculated from the Nicolsky–Eisenman equation. Dotted lines, asymptotes; numbers, detection limits expressed as concentration. SLS, standard leech saline.

Calibration solutions contained an ionic background (mM): KCl, 110; NaCl, 10; $MgCl_2$, 0·5; Hepes, 10; pH 7·3 adjusted with KOH. For Mg^{2+} calibration solutions, $MgCl_2$ was either omitted or added from a 1 M stock solution (Fluka) to final concentrations of 0·25, 0·5, 1, 2·5, 5 and 10 mM. For Na^+ calibration solutions, NaCl was either omitted or added from a stock solution to final concentrations of 1, 2·5, 5, 10, 25 and 50 mM. In addition, Ca^{2+} was buffered to a free concentration of 10^{-7} M by adding 0·727 mM $CaCl_2$ and 1 mM EGTA. pH calibration solutions were buffered either with 10 mM Hepes or 10 mM Mes (2-(*N*-morpholino)ethanesulphonic acid) to a pH of 7·67 and 6·22, respectively, by the addition of equal amounts of KOH.

In the Mg^{2+}- and Na^+-free calibration solutions it was assumed that the measuring ion concentration was $\leqslant 10^{-5}$ M. Calculations according to the Debye–Hückel equations (Ammann, 1986) showed that the single ion activity coefficients of Na^+ and Mg^{2+} in the respective calibration solutions changed less than 2% between the solution with the highest and the lowest measuring ion concentration. Therefore, no attempts were made to keep the ionic strength of the Na^+ and Mg^{2+} calibration solutions constant.

Measuring procedure

All potentials were measured against the potential of an extracellular reference electrode (agar bridge and Ag–AgCl cell), using two-channel voltmeters with differential amplifiers (WPI electrometer FD223; input resistance $10^{15}\,\Omega$; WPI, Mauer, Germany) and recorded continuously on a chart recorder (Gould, Dietzenbach, Germany).

Similar to Fry, Hall, Blatter & McGuigan (1990), we found that in contrast to the values for p(ion) (−log[ion]), the calculated ion concentrations were not normally distributed. Therefore, means ± S.D. are only given in connection with p(ion) values. Mean ion concentrations were calculated from the mean p(ion). The number of experiments n always refers to the number of experiments carried out on different preparations.

After every successful experiment, electrodes were recalibrated. While the two calibration curves for the Na^+ and the pH electrodes were usually in good agreement, most Mg^{2+} electrodes suffered from a considerable loss in sensitivity during an experiment. An experiment was used for quantitative evaluation when the values calculated from the two calibration curves did not differ by more than 0·4 mM (Buri & McGuigan, 1990). These were only about 30% of all experiments.

Determination of the rate of Mg^{2+} extrusion

Ion-sensitive microelectrodes measure changes in ion activities, but in the present study these changes are interpreted as ion fluxes across the cell membrane. It has to be kept in mind, however, that these changes might also be caused or modulated by release and/or uptake from intracellular stores.

To quantify the effects of experimental procedures (e.g. introduction of inhibitors, changes in the extracellular ionic environment) on Mg^{2+} regulatory processes, the 'rate of Mg^{2+} extrusion' (v_{extr}) after an evoked elevation of $[Mg^{2+}]_i$ was determined as $\Delta pMg_i\ min^{-1}$. For each preparation a control value $v_{extr,control}$ was determined, so that v_{extr} values under different experimental conditions could always be expressed as percentage of $v_{extr,control}$. These standardized values were used to determine mean results from different preparations.

Measurement of $[Ca^{2+}]_i$

The intracellular free calcium concentration ($[Ca^{2+}]_i$) was measured by the use of iontophoretically injected fura-2 (Molecular Probes, Eugene, OR, USA), as previously described in detail (Hochstrate & Schlue, 1994).

Control measurements were carried out by exposing the preparations to a bath solution containing 40 mM KCl. The resulting depolarization evoked a considerable increase in $[Ca^{2+}]_i$ which was compared with the response to a bath solution containing 30 mM $MgCl_2$.

Thermodynamic equilibrium of a hypothetical Na^+–Mg^{2+} exchanger

The thermodynamic equilibrium ($[Mg^{2+}]_{eq}$) of a hypothetical Na^+–Mg^{2+} antiporter, which exchanges x Na^+ ions for 1 Mg^{2+} ion was calculated from the following equation (Flatman & Smith, 1990):

$$[Mg^{2+}]_{eq} = [Mg^{2+}]_o \left(\frac{[Na^+]_i}{[Na^+]_o}\right)^x 10^{E_m(x-2)/58\,mV}.$$

RESULTS

Steady-state values of $[Mg^{2+}]_i$ and $[Na^+]_i$

In standard leech saline the mean $[Mg^{2+}]_i$ and $[Na^+]_i$ in the Retzius neurones of the leech *Hirudo medicinalis* were found to be 0·46 mM (pMg, 3·34 ± 0·23; range, 0·1–1·2 mM; $n = 32$) and 8·95 mM (pNa, 2·05 ± 0·15; range, 5·1–15·5 mM; $n = 21$), respectively, at a mean E_m of −35·6 ± 6·1 mV ($n = 32$).

If Mg^{2+} were passively distributed across the cell membrane, a $[Mg^{2+}]_i$ of 16·9 mM would be expected under the above conditions according to the Nernst equation. A 1 Na^+–1 Mg^{2+} antiport would theoretically reduce $[Mg^{2+}]_i$ to ⩾ 0·41 mM, a 2 Na^+–1 Mg^{2+} antiport to ⩾ 0·01 mM (for equation see Methods).

Effect of increasing $[Mg^{2+}]_o$ on $[Mg^{2+}]_i$, $[Na^+]_i$ and E_m

Simultaneous measurements of $[Mg^{2+}]_i$, $[Na^+]_i$ and E_m in Retzius neurones at a constant $[Na^+]_o$ of 45 mM (Na^+ partially substituted by $NMDG^+$ and Mg^{2+}, respectively), demonstrated that an increase in $[Mg^{2+}]_o$ (from 1 to 30 mM for 5 min) led to an increase in $[Mg^{2+}]_i$ (ΔpMg, −0·32 ± 0·18, $n = 35$), a decrease in $[Na^+]_i$ (ΔpNa, 0·15 ± 0·05, $n = 35$) and a membrane depolarization (ΔE_m, 2·0 ± 1·7 mV, $n = 35$). A subsequent reduction of $[Mg^{2+}]_o$ (back to 1 mM) had the opposite effect (Fig. 2).

The shifts in $[Mg^{2+}]_i$ were compatible with the changes in $[Mg^{2+}]_{eq}$ calculated for a 1 Na^+–1 Mg^{2+} antiport (dotted line in Fig. 2*A*). At the beginning of the experiment $[Mg^{2+}]_i$ was close to $[Mg^{2+}]_{eq}$. When $[Mg^{2+}]_o$ was increased, $[Mg^{2+}]_i$ increased towards the altered $[Mg^{2+}]_{eq}$. Unfortunately, it was not possible to wait and see whether $[Mg^{2+}]_i$ actually reached this value, as the cells did not survive such a long exposure to high $[Mg^{2+}]_o$. When

$[Mg^{2+}]_o$ was reduced to its original value, $[Mg^{2+}]_i$ again approached the calculated $[Mg^{2+}]_{eq}$. The experimental results, however, were incompatible with the values calculated for a 2 Na^+–1 Mg^{2+} antiport (dotted line in Fig. 2*B*).

Evidence for Na^+–Mg^{2+} antiport during inhibition of the Na^+–K^+ pump

Although opposing changes of $[Na^+]_i$ and $[Mg^{2+}]_i$ were observed in experiments as shown in Fig. 2, these changes differed significantly in quantity and time course, so that alternative explanations for the action of the postulated Na^+–Mg^{2+} antiport had to be considered. Thus, the observed $[Na^+]_i$ decrease in the presence of 30 mM $[Mg^{2+}]_o$ might be caused by a reduction of the Na^+ conductance of the cell membrane and a subsequent shift in the equilibrium of the Na^+–K^+ pump. To rule out this possibility, the Na^+–K^+ pump was blocked either by ouabain (0·5 mM) or by exposing the cell to a nominally K^+-free bath solution. In both cases $[Na^+]_i$ increased rapidly (Fig. 3). When, in addition, $[Mg^{2+}]_o$ was increased to 30 mM, the increase in $[Na^+]_i$ slowed down and, in some cases, finally reversed to a $[Na^+]_i$ decrease (Fig. 3*A*). This effect could be inhibited by the application of 2 mM amiloride (Fig. 3*B* and *C*), a concentration which also effectively inhibited Mg^{2+} extrusion from Mg^{2+}-loaded Retzius neurones (see below). A small, persisting reduction in $[Na^+]_i$ increase in the presence of 30 mM $[Mg^{2+}]_o$ and 2 mM amiloride probably reflects the reduction of the Na^+ conductance induced by high $[Mg^{2+}]_o$.

When $[Mg^{2+}]_o$ was reduced from 30 to 1 mM while the Na^+–K^+ pump was still inhibited, the rate of Mg^{2+} extrusion was reduced (Fig. 3*A*) presumably reflecting the dependence of the equilibrium concentration ($[Mg^{2+}]_{eq}$) on the Na^+ gradient.

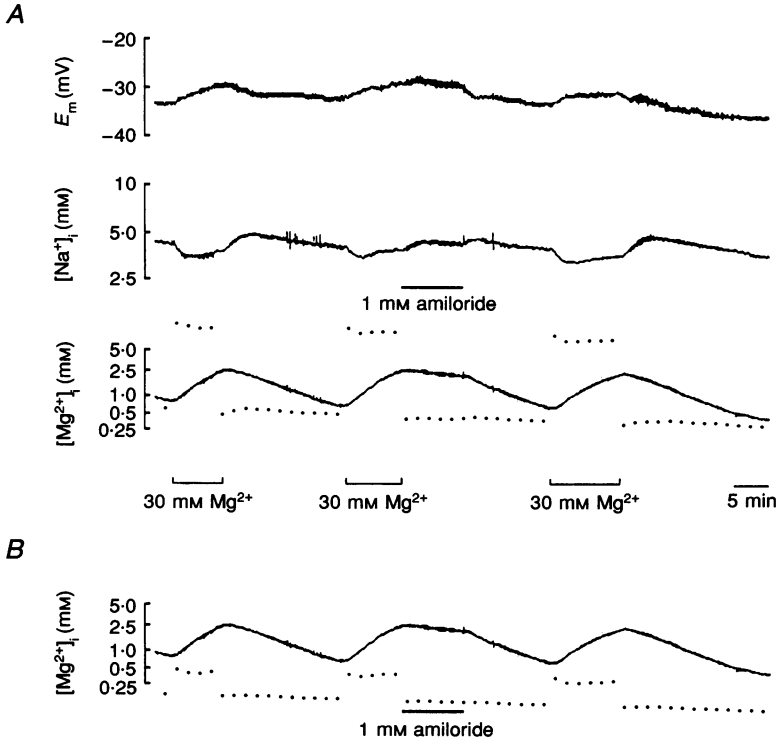

Figure 2. Simultaneous measurement of $[Na^+]_i$, $[Mg^{2+}]_i$ and E_m

A, during a simultaneous measurement of $[Na^+]_i$, $[Mg^{2+}]_i$ and E_m, $[Mg^{2+}]_o$ was increased from 1 to 30 mM while $[Na^+]_o$ remained constant at 45 mM. This led to an increase in $[Mg^{2+}]_i$, a decrease in $[Na^+]_i$ and a depolarization of the cell. A subsequent reduction of $[Mg^{2+}]_o$ back to 1 mM had opposite effects. Amiloride, 1 mM, inhibited all changes induced by the reduction of $[Mg^{2+}]_o$. Dots represent calculated values of $[Mg^{2+}]_{eq}$ for a 1 Na^+–1 Mg^{2+} antiport. *B*, same $[Mg^{2+}]_i$ trace as in *A*, but with $[Mg^{2+}]_{eq}$ (dots) calculated for a 2 Na^+–1 Mg^{2+} antiport. Only the values calculated for a 1 Na^+–1 Mg^{2+} antiport are compatible with the experimental data.

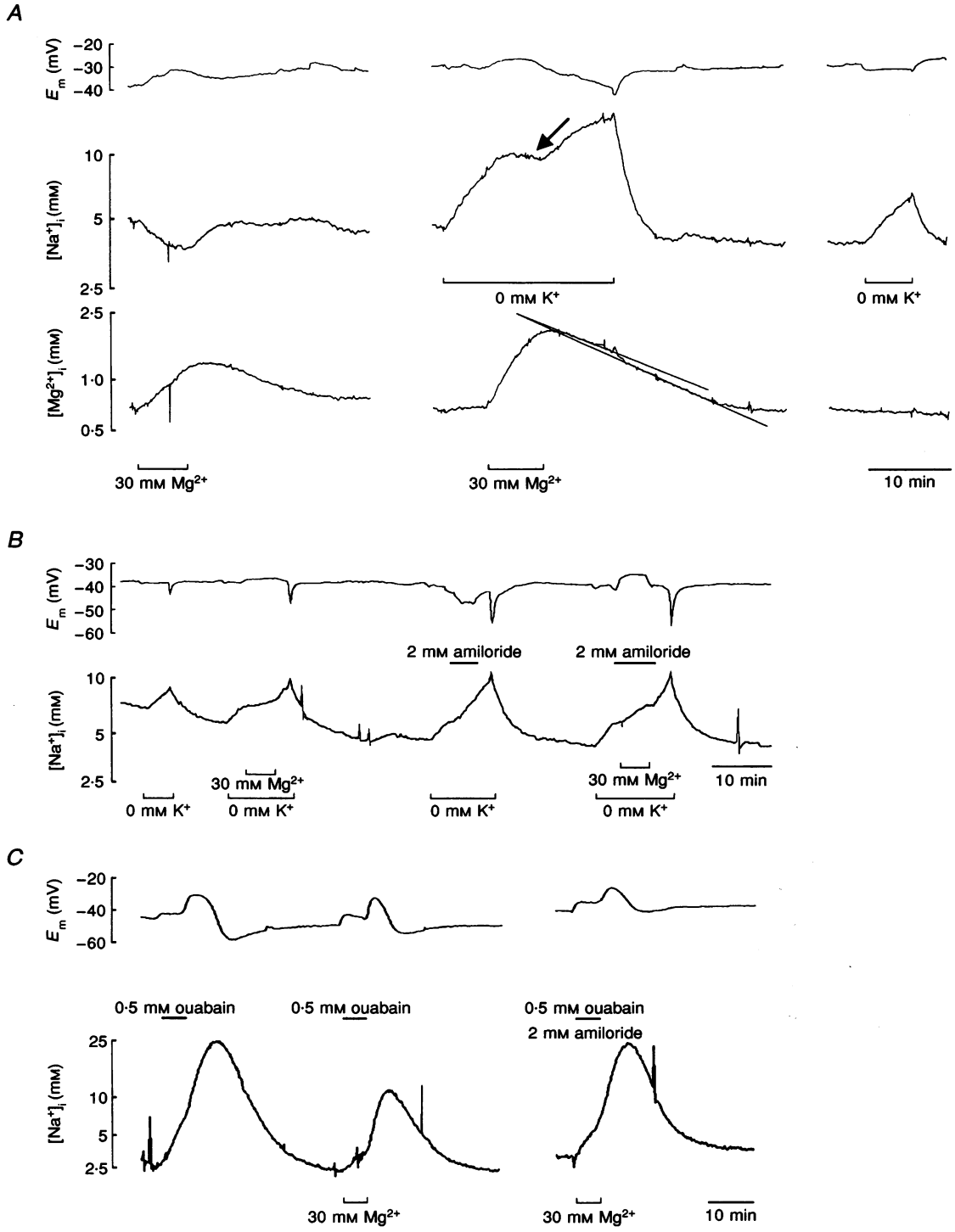

Figure 3. Inhibition of the Na^+–K^+ pump by ouabain or 0 $[K^+]_o$

A, when Retzius neurones were exposed to a K^+-free bath solution, $[Na^+]_i$ started to increase, due to the inhibition of the Na^+–K^+ pump. An additional increase in $[Mg^{2+}]_o$ to 30 mM slowed the increase of $[Na^+]_i$ considerably or even led to a decrease of $[Na^+]_i$ (arrow), while $[Mg^{2+}]_i$ increased. Returning to 1 mM $[Mg^{2+}]_o$ in the absence of $[K^+]_o$ resulted in a decrease of $[Mg^{2+}]_i$ and an increase of $[Na^+]_i$. Upon re-addition of $[K^+]_o$, $[Na^+]_i$ rapidly approached its original value while the decrease in $[Mg^{2+}]_i$ was accelerated (compare slope indicated by lines). *B*, in the absence of $[K^+]_o$ the effect of high $[Mg^{2+}]_o$ on $[Na^+]_i$ could be inhibited by 2 mM amiloride. *C*, similar experiment as in *A* and *B*, except that the Na^+–K^+ pump was inhibited by 0·5 mM ouabain.

Investigations on the reversibility of the postulated Na^+–Mg^{2+} antiport

Upon reversal of the Na^+ gradient (0 mM $[Na^+]_o$), a Na^+–Mg^{2+} antiporter should pump Mg^{2+} into the cell. However, the expected increase in $[Mg^{2+}]_i$ did not occur in these experiments (see Fig. 4). In the absence of $[Na^+]_o$, an increase of $[Mg^{2+}]_o$ (from 1 to 30 mM) caused an increase of $[Mg^{2+}]_i$. This increase, however, was even slower than in the presence of 45 mM extracellular Na^+ and amounted to 74·3 ± 17·4% ($n = 4$) of the control value. After reducing $[Mg^{2+}]_o$ from 30 to 1 mM in the absence of external Na^+, $[Mg^{2+}]_i$ did not change at all, but remained at its elevated level. Only after re-addition of $[Na^+]_o$, did $[Mg^{2+}]_i$ decrease towards its original value.

In contrast to the control experiments in the presence of $[Na^+]_o$, an increase in $[Mg^{2+}]_o$ in the absence of $[Na^+]_o$ did not elicit an additional decrease in $[Na^+]_i$. These results led to the conclusion that the Na^+–Mg^{2+} antiporting system might be inhibited in the absence of $[Na^+]_o$ or, as 0 $[Na^+]_o$ induced a rapid decrease in $[Na^+]_i$ to values below 1 mM, by low $[Na^+]_i$.

Dependence of the rate of Mg^{2+} extrusion on $[Na^+]_o$

To gain further insight into this inhibitory effect, Mg^{2+} extrusion at various $[Na^+]_o$ levels was investigated (Fig. 5). To this end, Retzius neurones were loaded with Mg^{2+} by exposing them to very high (50 mM) $[Mg^{2+}]_o$. In this solution the total Na^+ concentration was reduced to 16·5 mM to maintain osmolarity. Correspondingly, a considerable decrease in $[Na^+]_i$ (and an initial hyperpolarization due to this Na^+ efflux) was observed in parallel to the rise in $[Mg^{2+}]_i$ (Fig. 5*A*). When $[Mg^{2+}]_o$ was again reduced to 1 mM, $[Na^+]_o$ was simultaneously changed to various levels (10, 30, 50, 75 and 100% of the standard $[Na^+]_o$ of 90 mM, Fig. 5*A* and *B*). $[Na^+]_i$ then rapidly approached a plateau value while the concomitant Mg^{2+} extrusion was slow, so that the Na^+ gradient was constant during most of the Mg^{2+}-regulation period. Although the equilibrium values of $[Mg^{2+}]_{eq}$ (dots in Fig. 5*A*) were well below the actual $[Mg^{2+}]_i$, Mg^{2+} extrusion was very slow and only recovered its original value $v_{extr,control}$ when the standard total $[Na]_o$ of 90 mM was restored. As shown in Fig. 5*B* and *C*, v_{extr} depended strictly on the extracellular (and/or intracellular) Na^+ concentration.

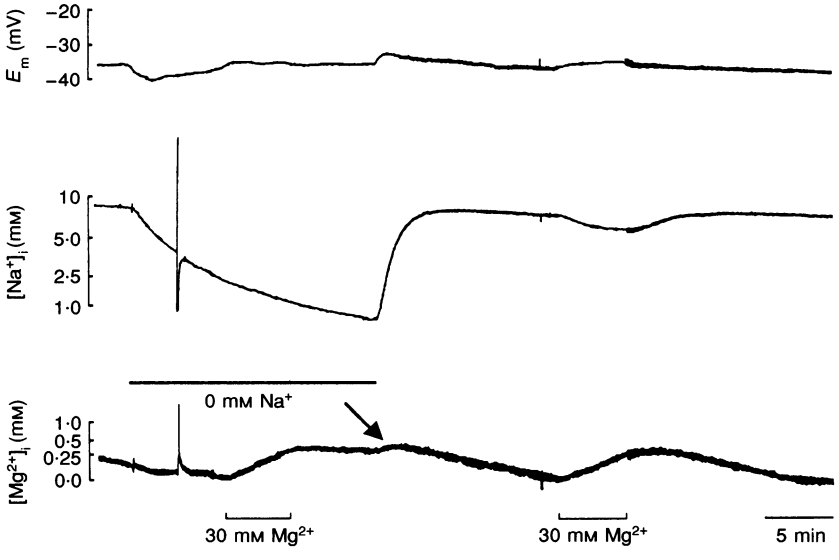

Figure 4. Inhibition of Na^+–Mg^{2+} antiport by 0 $[Na^+]_o$

During simultaneous measurement of $[Na^+]_i$, $[Mg^{2+}]_i$ and E_m, $[Mg^{2+}]_o$ was varied between 1 and 30 mM while $[Na^+]_o$ was either completely absent or remained constant at 45 mM. In 0 mM $[Na^+]_o$ with 1 mM $[Mg^{2+}]_o$, $[Mg^{2+}]_i$ remained low. $[Mg^{2+}]_i$ increased in 0 mM $[Na^+]_o$ with 30 mM $[Mg^{2+}]_o$, but at a lower rate than at 45 mM $[Na^+]_o$ and without being accompanied by a reduction in $[Na^+]_i$. Upon returning to 0 mM $[Na^+]_o$with only 1 mM $[Mg^{2+}]_o$, $[Mg^{2+}]_i$ seemed to decrease slightly in parallel to the decrease of $[Na^+]_i$. This apparent decrease is probably due to interference of Na^+ at the Mg^{2+}-sensitive electrode, as, after the re-addition of $[Na^+]_o$, $[Mg^{2+}]_i$ seemed to increase rapidly (arrow) before it started to decrease towards its original value.

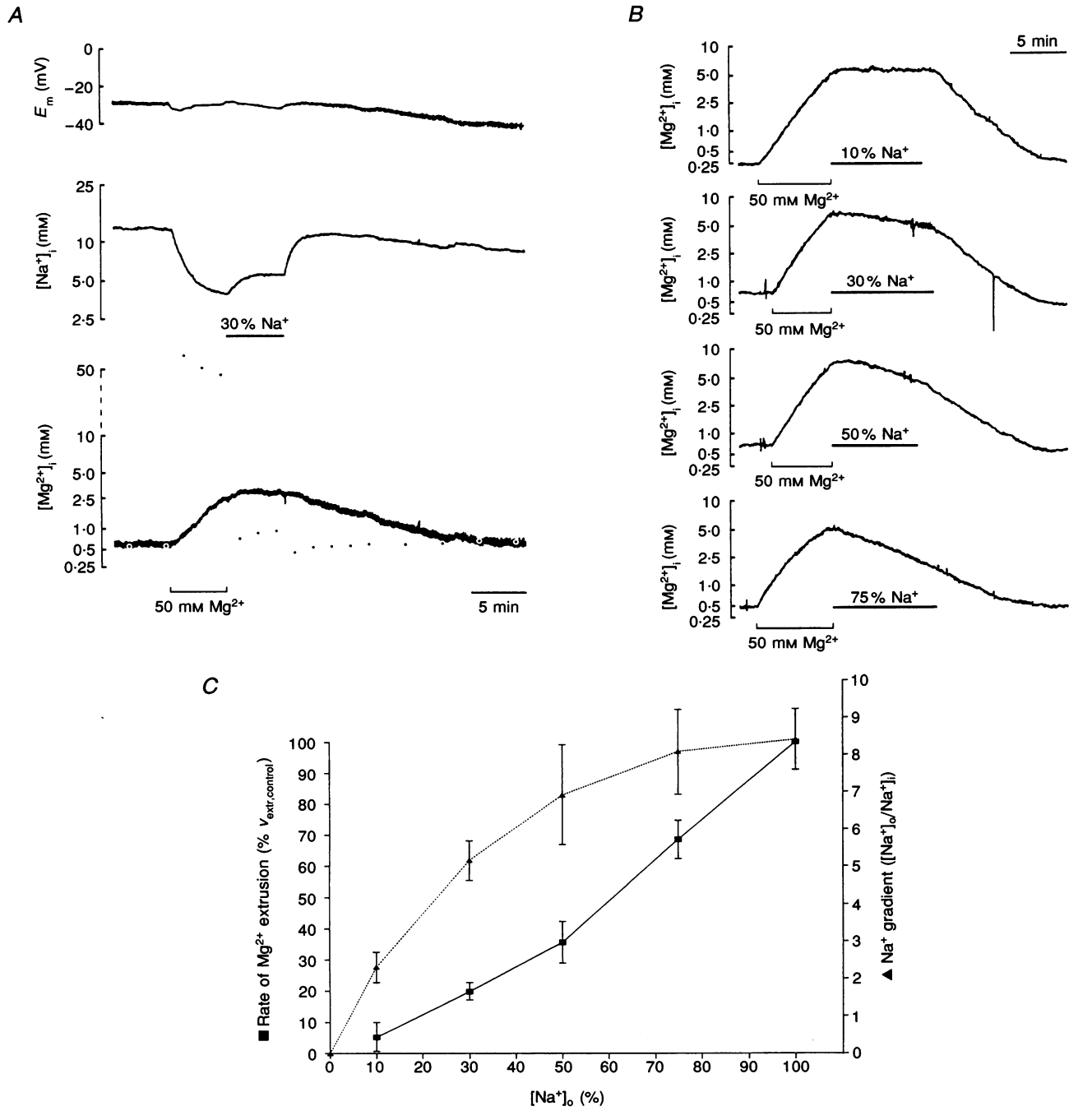

Figure 5. $[Na^+]_o$-dependent inhibition of Mg^{2+} extrusion

A, when exposed to a bath solution containing 50 mM Mg^{2+} and 16·5 mM Na^+, $[Mg^{2+}]_i$ in the Retzius neurones increased, while $[Na^+]_i$ decreased considerably. During a subsequent exposure of the cell to 1 mM $[Mg^{2+}]_o$ but only 30% (27 mM) of the standard $[Na^+]_o$, $[Na^+]_i$ increased rapidly to a plateau value, whereas $[Mg^{2+}]_i$ did not change at all. As in Fig. 2, dots represent calculated values of $[Mg^{2+}]_{eq}$ for a 1 Na^+–1 Mg^{2+} antiport. Even at 30% of the standard $[Na^+]_o$, $[Mg^{2+}]_{eq}$ is well below the actual $[Mg^{2+}]_i$, so that a decrease in $[Mg^{2+}]_i$ should be expected. However, $[Mg^{2+}]_i$ only started to decrease when the standard $[Na^+]_o$ was restored. *B*, again cells were loaded with Mg^{2+} by exposing them to a $[Mg^{2+}]_o$ of 50 mM. Upon returning to 1 mM $[Mg^{2+}]_o$, $[Na^+]_o$ was changed to 10, 30, 50 or 75% (9, 27, 45 or 67·5 mM) of the standard $[Na^+]_o$. Under these conditions, the rate of Mg^{2+} extrusion depended on $[Na^+]_o$. *C*, the rate of Mg^{2+} extrusion (v_{extr}, ■) and the Na^+ gradient ($[Na^+]_o/[Na^+]_i$, ▲) were plotted against $[Na^+]_o$ (means ± S.D. of 4 experiments). Between 50 and 100% $[Na^+]_o$, the Na^+ gradient decreased only to about 80%. Hence, in this range the large reduction of v_{extr} cannot be explained by the reduction in the Na^+ gradient. Only below 30% $[Na^+]_o$ did a considerable reduction in the Na^+ gradient occur which was likely to affect v_{extr}.

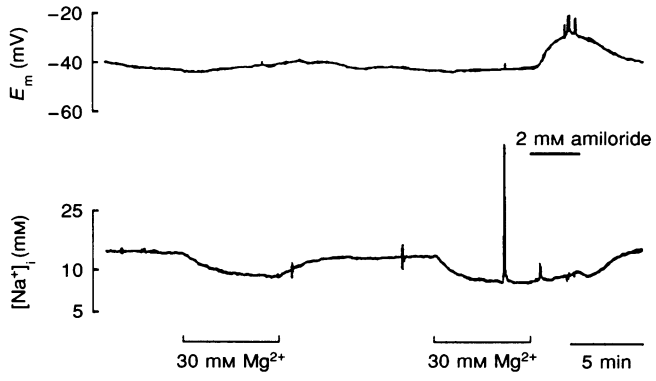

Figure 6. Inhibition of Na^+ influx by amiloride

As in Fig. 2, elevation of $[Mg^{2+}]_o$ from 1 to 30 mM leads to a decrease in $[Na^+]_i$, while a reduction of $[Mg^{2+}]_o$ from 30 to 1 mM evokes a Na^+ influx ($[Na^+]_o$ was held at 45 mM during the whole experiment). The Na^+ influx was inhibited by 2 mM amiloride. This inhibition suggests that Mg^{2+} and Na^+ fluxes across the cell membrane are coupled.

To demonstrate further that the reduction in v_{extr} was not simply due to a reduction of the Na^+ gradient across the cell membrane, the Na^+ gradient ($[Na^+]_o/[Na^+]_i$) was calculated from the plateau value of $[Na^+]_i$ (and the known $[Na^+]_o$) and plotted against $[Na^+]_o$. Figure 5*C* shows that the Na^+ gradient between 50 and 100% $[Na^+]_o$ was only reduced to about 80%, while the rate of Mg^{2+} extrusion was reduced to about 35% of its maximum speed. In this range a reduction of the Na^+ gradient cannot be responsible for the large decrease in v_{extr}. Only below 30% $[Na^+]_o$ is the Na^+ gradient so strongly reduced that an influence on v_{extr} is expected.

Effect of amiloride

Mg^{2+} extrusion from the Retzius neurones could be inhibited by amiloride (Fig. 2). Inhibition was dose dependent, with 50% inhibition (K_i) occurring at an amiloride concentration of 0·25 mM and a Hill coefficient of 1·3 (data from 8 experiments). At a concentration of 2 mM, Mg^{2+} extrusion was almost completely inhibited (92·2 ± 8·2%). Amiloride not only blocked Mg^{2+} extrusion, but also the parallel Na^+ influx. Again, this inhibition was dose dependent (compare Figs 2 and 6). This is compatible with the idea that the Mg^{2+} and Na^+ movements across the cell membrane are coupled.

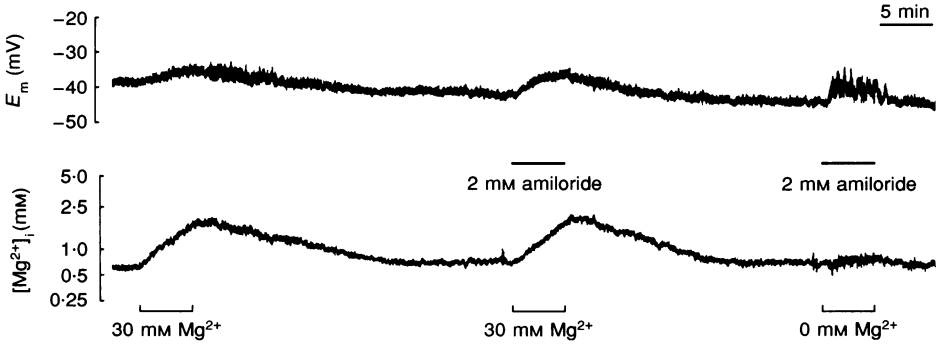

Figure 7. No inhibition of Mg^{2+} influx by amiloride

In contrast to Mg^{2+} extrusion, the increase in $[Mg^{2+}]_i$ in the presence of 30 mM $[Mg^{2+}]_o$ was not inhibited by 2 mM amiloride. Exposing the cell to 2 mM amiloride in the absence of $[Mg^{2+}]_o$ demonstrates that amiloride does not show any direct interference at the Mg^{2+}-sensitive electrode. It should be noted that exposure of Retzius neurones to a 0 mM $[Mg^{2+}]_o$ solution for up to 10 min did not decrease $[Mg^{2+}]_i$ significantly (not shown).

However, when the neurones were exposed to 30 mM $[Mg^{2+}]_o$, the resulting Mg^{2+} influx was not inhibited by 2 mM amiloride (Fig. 7, $n = 7$).

The amiloride derivative 5-(*N*-ethyl-*N*-isopropyl)-amiloride (EIPA) had no effect on Mg^{2+} influx or efflux at concentrations up to 2 mM. Imipramine and quinidine could not be used in our experiments, as they caused very strong interference at the Mg^{2+} electrode.

Interactions between $[Mg^{2+}]_i$ and pH_i

Experiments were carried out to investigate possible interactions between $[Mg^{2+}]_i$ and pH_i. In one set of experiments the effect of variation in pH_i on $[Mg^{2+}]_i$ was investigated. To this end, Retzius neurones were exposed to HCO_3^--containing leech saline equilibrated with 5% CO_2 (in O_2). This caused a rapid, transient acidification of the neurones (0·27 ± 0·08 pH units, $n = 12$). Upon removal of the CO_2, considerable alkalinization occurred (0·42 ± 0·19 pH units, $n = 9$) after which pH_i slowly approached its original value. During acidification some Retzius neurones showed a slight decrease in $[Mg^{2+}]_i$ (mean of all experiments: ΔpMg 0·08 ± 0·08, $n = 10$), while during alkalinization a minor increase in $[Mg^{2+}]_i$ was observed occasionally (mean of all experiments: ΔpMg, −0·06 ± 0·06, $n = 8$). Neither change in $[Mg^{2+}]_i$ was significant.

In another series of experiments, pH_i was measured while $[Mg^{2+}]_o$ was varied between 1 and 30 mM, under conditions where $[Na^+]_o$ was maintained as described above (cf. Fig. 2). Under these conditions, no effect on pH_i could be detected during the increase of $[Mg^{2+}]_i$. During the decrease of $[Mg^{2+}]_i$ after $[Mg^{2+}]_o$ was reduced from 30 to 1 mM, a slight acidification of the Retzius neurones could occasionally be observed. The mean change in pH_i (ΔpH_i, −0·013 ± 0·02, $n = 10$) in all experiments, however, was not significant.

In additional control experiments the effect of amiloride on pH_i was investigated. During a 5 min application of 2 mM amiloride in Hepes buffered solution pH_i decreased by 0·12 ± 0·01 pH units ($n = 3$), confirming that in the nominal absence of HCO_3^- the pH_i in leech Retzius neurones is mainly regulated by an amiloride-sensitive Na^+–H^+ antiport (Schlue & Thomas, 1985).

Effect of increased $[Cl^-]_o$ in high $[Mg^{2+}]_o$ solutions

When Mg^{2+} was increased in the bath solution, $MgCl_2$ was substituted for NMDG-Cl to maintain osmolarity. However, this resulted in an increase of the Cl^- concentration ($[Cl^-]_o$) from 95 mM in a solution containing 1 mM $MgCl_2$ to 153 mM in a solution containing 30 mM $MgCl_2$. To exclude possible effects of the elevated $[Cl^-]_o$, control experiments were carried out in which the excess Cl^- was substituted either by SO_4^{2-} or by gluconate. No differences were found in the responses of the Retzius neurones to these three bath solutions ($n = 4$).

Influence of elevated $[Mg^{2+}]_i$ on the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$)

The influence of elevated $[Mg^{2+}]_i$ on $[Ca^{2+}]_i$ was investigated using the fluorescence probe fura-2. A rise in $[Ca^{2+}]_i$ might be sensed by Mg^{2+}- and Na^+-sensitive microelectrodes, because they suffer considerably from interference by Ca^{2+} ions (Günzel & Galler, 1991 and Schlue, 1991, respectively). Therefore, changes in $[Ca^{2+}]_i$ may be misinterpreted as changes in $[Mg^{2+}]_i$ or $[Na^+]_i$, respectively. However, when $[Mg^{2+}]_i$ was increased by raising $[Mg^{2+}]_o$ from 1 to 30 mM, no change in $[Ca^{2+}]_i$ was detected ($n = 3$).

DISCUSSION

In all preparations investigated so far, Mg^{2+} is not distributed passively across the cell membrane. However, the mechanisms of Mg^{2+} extrusion are still controversial. Many authors describe Na^+–Mg^{2+} antiporting systems, but these antiports may or may not be ATP dependent. The stoichiometry of Na^+–Mg^{2+} antiports investigated so far ranges from 1 Na^+–1 Mg^{2+} to 3 Na^+–1 Mg^{2+} in different preparations.

Steady-state values for $[Mg^{2+}]_i$ in leech Retzius neurones

In Retzius neurones of the leech *Hirudo medicinalis*, $[Mg^{2+}]_i$ was found to be 0·46 mM. This value is in the same range as values published for *Helix* neurones (0·66 mM, Alvarez-Leefmans, Gamiño & Rink, 1984) and for smooth muscle (0·1–0·5 mM, McGuigan *et al.* 1991), but somewhat lower than values published for skeletal muscle (about 1 mM, McGuigan *et al.* 1991).

At a mean E_m of −35·6 mV and a $[Mg^{2+}]_o$ of 1 mM, a $[Mg^{2+}]_i$ of 16·9 mM would be expected if Mg^{2+} were passively distributed across the cell membrane. It is therefore concluded that transport mechanisms must exist in the Retzius neurones which actively extrude Mg^{2+} from the cytoplasm.

Evidence for the existence of Na^+–Mg^{2+} antiport in leech Retzius neurones

The most likely candidate for a Mg^{2+} transport mechanism in leech Retzius neurones was the Na^+–Mg^{2+} antiport, as evidence for Na^+-dependent Mg^{2+} extrusion had already been demonstrated for several cell types (for review see Beyenbach, 1990). Simultaneous measurements of $[Mg^{2+}]_i$, $[Na^+]_i$ and E_m in Retzius neurones showed that changing $[Mg^{2+}]_o$ elicited changes in $[Mg^{2+}]_i$, $[Na^+]_i$ and E_m. These results would be expected if Na^+–Mg^{2+} antiport was present in these cells (Fig. 2). However, two findings seem to argue against the existence of Na^+–Mg^{2+} antiport: (1) the quantity and the time course of the changes in $[Mg^{2+}]_i$ and $[Na^+]_i$ differed significantly and (2) the antiport could not be reversed by reversing the Na^+ gradient, i.e. no

increase in $[Mg^{2+}]_i$ was measured in Na^+-free bath solutions. Both findings will now be discussed in detail.

Time course of $[Na^+]_i$ and $[Mg^{2+}]_i$ changes

If both $[Na^+]_i$ and $[Mg^{2+}]_i$ were predominantly regulated by a Na^+–Mg^{2+} antiport, an increase in $[Mg^{2+}]_o$ should induce an increase in $[Mg^{2+}]_i$ and a decrease in $[Na^+]_i$ of identical quantity and time course. This is not the case: $[Na^+]_i$ is predominately regulated by the Na^+–K^+ pump and $[Mg^{2+}]_i$, apart from being regulated by the Na^+–Mg^{2+} antiport, is well buffered and may even be sequestered by intracellular organelles. It is not surprising, therefore, that overall changes in $[Mg^{2+}]_i$ and $[Na^+]_i$, as measured by an ion-sensitive microelectrode, do not match precisely. In the recordings shown in Fig. 2, initial $[Mg^{2+}]_i$ increase is slower than the accompanying $[Na^+]_i$ decrease. $[Mg^{2+}]_i$ then continues to rise while $[Na^+]_i$ remains unchanged after the initial decrease or even starts to increase again. Analogous differences in the time course are observed when $[Mg^{2+}]_o$ is changed back to the original low level. While the initial low rate of $[Mg^{2+}]_i$ changes might reflect a high intracellular buffering capacity, the changes in $[Na^+]_i$ are probably dominated by the Na^+–K^+ pump: (1) the fast initial change might be caused by inhibition of Na^+ channels through high $[Mg^{2+}]_o$ (Sheets & Hanck, 1992), which should lead to a more efficient Na^+ extrusion through the Na^+–K^+ pump: (2) the Na^+–K^+ pump might then stabilize $[Na^+]_i$ near its original level. Experiments during which the Na^+–K^+ pump was inhibited either by ouabain or by K^+-free bath solutions, support these assumptions. When $[Mg^{2+}]_o$ was increased during pump inhibition, the corresponding changes in $[Na^+]_i$ were superimposed on a general increase in $[Na^+]_i$. Under these conditions the time course of these Mg^{2+}-induced changes in $[Na^+]_i$ paralleled the time course of the $[Mg^{2+}]_i$ changes more closely. The $[Na^+]_i$ changes were sensitive to amiloride which supports the assumption that they are caused by a reversed action of a Na^+–Mg^{2+} antiporter. The remaining reduction of the Na^+ conductance in the presence of 30 mM $[Mg^{2+}]_o$ and 2 mM amiloride may reflect the postulated direct inhibition of Na^+ channels at high $[Mg^{2+}]_o$.

Reversibility of the Na^+–Mg^{2+} antiport and inhibition of the antiport in low $[Na^+]_o$

In the presence of $[Na^+]_o$ the Na^+–Mg^{2+} antiport in leech Retzius neurones showed reversal (see Figs 2 and 3); an increase in $[Mg^{2+}]_o$ with $[Na^+]_o$ held at 45 mM led to an increase in $[Mg^{2+}]_i$, a decrease in $[Na^+]_i$ and a depolarization of the neurones.

In contrast to these results, no reversal of the Na^+–Mg^{2+} antiport was observed after the removal of Na^+ from the bathing medium. In the absence of $[Na^+]_o$, a massive influx of Mg^{2+} into the neurones should occur. Such an influx could never be observed.

In the presence of high $[Mg^{2+}]_o$ an increase in $[Mg^{2+}]_i$ was observed. However, in the absence of extracellular Na^+ this increase was even slower than in the presence of extracellular Na^+, and it was not accompanied by an additional decrease in $[Na^+]_i$.

Upon returning to low $[Mg^{2+}]_o$, Mg^{2+} extrusion was inhibited. As illustrated in Fig. 4, interpretation of these experiments was complicated by interference through Na^+ at the Mg^{2+}-sensitive microelectrode. In Na^+-free bath solutions, $[Na^+]_i$ decreased to values below 1 mM. Usually the Mg^{2+} electrode responded to this decrease with a drop in potential of 1–2 mV which might be misinterpreted as a decrease in $[Mg^{2+}]_i$. Thus, in the absence of $[Na^+]_o$, $[Mg^{2+}]_i$ seemed to decrease slightly in parallel to the decrease of $[Na^+]_i$. After the re-addition of $[Na^+]_o$, $[Mg^{2+}]_i$ seemed to increase rapidly (arrow in Fig. 4) before it started to decrease towards its original value. If the recording of $[Mg^{2+}]_i$ is corrected for the interference from Na^+, $[Mg^{2+}]_i$ does not decrease at all in the absence of $[Na^+]_o$, indicating a complete inhibition of the Na^+–Mg^{2+} antiport under these conditions.

On the basis of these results we conclude that the Na^+–Mg^{2+} antiport might be inhibited at low $[Na^+]_o$, and that the Mg^{2+} influx observed at high $[Mg^{2+}]_o$–0 $[Na^+]_o$ represents the passive portion of Mg^{2+} influx ('leakage'). This conclusion is supported by the fact that the rate of Mg^{2+} extrusion appears to be correlated to $[Na^+]_o$. There are two possible explanations of how the inhibition by low $[Na^+]_o$ could be mediated: either by extracellular Na^+ itself (e.g. at an allosteric binding site on the extracellular portion of the antiporter as suggested by Flatman & Smith, 1990), or by a relatively low affinity of the intracellular binding site for Na^+. When $[Na^+]_o$ is reduced to 50%, $[Na^+]_i$ decreases by an almost proportional amount, so that the Na^+ gradient (and therefore, the driving force of the Na^+–Mg^{2+} antiport) is reduced by less than 20% (Fig. 5*C*). Although this reduction should hardly effect Mg^{2+} extrusion, v_{extr} is actually reduced to about 35% of $v_{extr,control}$. In Na^+-free saline, $[Na^+]_i$ is near or even below 1 mM. This concentration might be too low to drive the antiport at a rate that can be detected within our experimental regime.

Stoichiometry of the Na^+–Mg^{2+} antiport in leech Retzius neurones

The simultaneous measurement of $[Mg^{2+}]_i$, $[Na^+]_i$ and E_m in the present experiments allowed us to calculate Mg^{2+} equilibrium values of hypothetical Na^+–Mg^{2+} antiporters using different stoichiometries, to compare these values with the actual values of $[Mg^{2+}]_i$, and to relate $[Mg^{2+}]_{eq}$ to the time course of $[Mg^{2+}]_i$ changes during the experimental recordings. Only the values obtained for a 1 Na^+–1 Mg^{2+} antiport were compatible with the experimental

observations (Figs 2 and 5A). Yet, it has to be kept in mind that a Na^+–Mg^{2+} antiport probably always has to work against a steady, passive influx of Mg^{2+} ions and that it never works with 100% efficiency. Therefore, the calculated values of $[Mg^{2+}]_{eq}$ should always be below and not (as e.g. shown in Fig. 5A) equal to the actual $[Mg^{2+}]_i$. However, due to various sources of error at the ion-sensitive microelectrodes, the values of both $[Mg^{2+}]_i$ and $[Mg^{2+}]_{eq}$ should rather be regarded as a range of values, the width of which is given by the discrepancy between the calibration curves of the ion-sensitive microelectrodes before and after an experiment (see Methods for acceptable limit). Hence, we do not think that results as shown in Fig. 5A are in contradiction to the existence of a 1 Na^+–1 Mg^{2+} antiport in the neuronal membrane.

A 1 Na^+–1 Mg^{2+} antiport is electrogenic and should, therefore, hyperpolarize the membrane during Mg^{2+} extrusion and depolarize the membrane when working in the reverse mode. These expected membrane potential changes were indeed observed in our experiments (Fig. 2). Furthermore, the electrogeneity of the Na^+–Mg^{2+} antiport in leech Retzius neurones is supported by recent voltage-clamp experiments (Müller, Günzel & Schlue, 1995). These experiments demonstrate that $[Mg^{2+}]_{eq}$, and thus the rate of Mg^{2+} extrusion, can be influenced by changing the neuronal membrane potential.

ATP dependence of the Na^+–Mg^{2+} antiport

Two types of ATP dependencies have been proposed for Na^+–Mg^{2+} antiporting systems: a direct coupling of the Na^+ and Mg^{2+} transport to ATP splitting (Frenkel *et al.* 1989) and an activation of the carrier protein by phosphorylation (DiPolo & Beaugé, 1988; Günther *et al.* 1990).

Calculations of $[Mg^{2+}]_{eq}$ for a Na^+–Mg^{2+} antiport demonstrate that intracellular Mg^{2+} levels can be maintained by a 1 Na^+ : 1 Mg^{2+} exchange, so that from an energetic point of view, ATP splitting should not be necessary for Mg^{2+} homeostasis.

In the presence of Na_o^+ the Na^+–Mg^{2+} antiport in leech Retzius neurones showed reversal (see Figs 2 and 3). An increase in $[Mg^{2+}]_o$ under maintenance of $[Na^+]_o$ led to an increase in $[Mg^{2+}]_i$, a decrease in $[Na^+]_i$ and a depolarization of the neurones. This reversibility of the Na^+–Mg^{2+} antiport argues against the presence of an ATP-driven Mg^{2+} pump which would still work in the 'forward' direction under the given conditions ($[Mg^{2+}]_o$, 1 mM; $[Na^+]_o$, 45 mM; $[Na^+]_i$ and E_m as measured during the experiment; ΔG_{ATP} ~55 kJ mol^{-1}; Läuger, 1991).

In summary, our results yield no evidence that the Na^+–Mg^{2+} antiport in leech Retzius neurones needs ATP splitting as an energy source; however, modulation of the activity of the antiporter by phosphorylation as suggested by Günther *et al.* (1990) cannot be excluded.

Inhibition of the Na^+–Mg^{2+} antiport by amiloride

Mg^{2+} extrusion was inhibited by amiloride, with 50% inhibition occurring at a concentration of 0·25 mM amiloride. This value is similar to those published for erythrocytes of various species (human: 0·4 mM, Lüdi & Schatzmann, 1987; chicken and rat: 0·4 and 0·68 mM, respectively, Günther *et al.* 1989; ferret: 0·5–1 mM, Flatman & Smith, 1990). In contrast to the investigations of Flatman & Smith (1990), who found that 2 mM amiloride inhibited transport by only 65% in ferret erythrocytes, but in accordance with the results of Lüdi & Schatzmann (1987) in human erythrocytes, Mg^{2+} extrusion in leech Retzius neurones was blocked almost completely by 2 mM amiloride.

As would have to be expected for a coupled Na^+–Mg^{2+} antiport, amiloride not only blocked Mg^{2+} transport but also the concomitant Na^+ fluxes. This was demonstrated both in the absence, and in the presence of, additional inhibition of the Na^+–K^+ pump (see above).

When the Na^+–K^+ pump was not blocked, the inhibitory effect of amiloride on the Na^+ influx during Mg^{2+} extrusion could not be investigated quantitatively. In leech Retzius neurones, amiloride can evoke considerable depolarizations (see e.g. Fig. 6) which are probably due to an inhibition of K^+ channels (Klusemann, Hintz & Schlue, 1994). The depolarizations induced the cells to generate action potentials. The Na^+ influx caused by these action potentials masked the inhibitory effect of amiloride on the Mg^{2+}-coupled Na^+ influx. However, in those cases, in which the depolarization was not strong enough to elicit spike generation, a dose-dependent inhibitory effect of amiloride on the Na^+ influx could be observed (e.g. compare the effect of 1 mM amiloride in Fig. 2 and 2 mM amiloride in Fig. 6).

In contrast to Mg^{2+} efflux, Mg^{2+} influx was not inhibited by amiloride. Together with the absence of an inhibition of Mg^{2+} influx in Na^+-free bath solutions this is an indication that most of the Mg^{2+} influx occurs through passive pathways, not through reversal of the Na^+–Mg^{2+} antiport.

Interactions between pH_i and $[Mg^{2+}]_i$

In the literature two types of interactions between pH_i and $[Mg^{2+}]_i$ have been described: (1) modification of pH_i regulation by changes in $[Mg^{2+}]_i$ in barnacle muscle fibres (Russell & Brodwick, 1988), and (2) modulation of $[Mg^{2+}]_i$ by changes in pH_i in cultured chicken heart cells (Freudenrich, Murphy, Levy, London & Lieberman, 1992), e.g. an increase in $[Mg^{2+}]_i$ induced by acidification.

We investigated in our preparation, whether the amiloride-induced acidification might cause changes in $[Mg^{2+}]_i$ or whether amiloride had a direct effect on $[Mg^{2+}]_i$ regulation. However, in control experiments no interaction between pH_i and $[Mg^{2+}]_i$ could be observed, indicating that amiloride affects the Na^+–Mg^{2+} exchanger directly.

Possible Ca^{2+} interference at the Na^+- and Mg^{2+}-sensitive microelectrode

Both the Na^+- and the Mg^{2+}-sensitive microelectrodes used in the present study are prone to interference through Ca^{2+}. Therefore, control experiments have to be carried out if $[Ca^{2+}]_i$ changes are to be expected during the experimental procedures. In the present study, the critical experiments were those with exposure of the neurones to Na^+-free bath solutions and the increase in $[Mg^{2+}]_i$ elicited by high $[Mg^{2+}]_o$.

In the absence of extracellular Na^+ an increase in $[Ca^{2+}]_i$ would have to be expected if a Na^+–Ca^{2+} antiport were present in leech Retzius neurones. However, Hochstrate & Schlue (1994) failed to induce an increase in $[Ca^{2+}]_i$ when exposing Retzius neurones to Na^+-free bath solutions.

Mg^{2+} and Ca^{2+} compete at many intracellular binding sites (Flatman, 1984). Therefore, an increase in $[Mg^{2+}]_i$ should lead to an increase in $[Ca^{2+}]_i$ and vice versa. However, control measurements showed no increase in $[Ca^{2+}]_i$ when $[Mg^{2+}]_i$ was increased by exposing the neurones to high $[Mg^{2+}]_o$, while $[Ca^{2+}]_i$ readily increased when the neurones were depolarized in a bath solution containing 40 mM K^+.

Physiological significance of Mg^{2+} regulation in the central nervous system

In the present study we present evidence for intracellular Mg^{2+} regulation by Na^+–Mg^{2+} antiport in identified neurones of an invertebrate central nervous system. Most studies of intracellular Mg^{2+} regulation have been carried out on muscle tissue and erythrocytes, while there are hardly any studies on nervous tissues, although the understanding of Mg^{2+} regulation in the central nervous system is of great importance. Low $[Mg^{2+}]_o$ can elicit epileptiform activity and spreading depression (Mody, Lambert & Heinemann, 1987). Mg^{2+} is also thought to play a key role in depressive states (Ananth & Yassa, 1979). This is strengthened by the findings that certain antidepressant drugs are able to inhibit Na^+–Mg^{2+} exchange (Féray & Garay, 1988).

Leech ganglia may provide an ideal model system for further investigation of Mg^{2+} regulation in the central nervous system because both neurones and glial cells are large, easily accessible and individually identifiable.

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