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FLUOROMETRIC EVIDENCE FOR DIFFERENT STOICHIOMETRIES FOR THE Na^+/Mg^{2+} EXCHANGE IN Mg-LOADED RAT THYMOCYTES

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1. ABSTRACT

The regulation of the cytosolic free magnesium concentration ($[Mg^{2+}]_i$) is a fundamental cellular process that requires magnesium extruding mechanisms. Here, we present evidence indicating that rat thymocytes are endowed with different Na/Mg exchange systems. Fluxes of magnesium were measured using the fluorescent magnesium indicator magfura-2. Cells were loaded with magnesium using the calcium ionophore A-23187 to 0.6-8.0 mM $[Mg^{2+}]_i$ (resting $[Mg^{2+}]_i = 0.38 \pm 0.06$ mM, n = 5). The presence of extracellular sodium was required for magnesium exit. The initial rate of $[Mg^{2+}]_i$ was stimulated by extracellular sodium with Michaelis-Menten kinetics. The Vmax of the sodium-dependent magnesium exit was markedly increased by $[Mg^{2+}]_i$. Holding the membrane potential at either –84 mV or at –10 mV had different effects on the sodium-stimulated magnesium efflux, depending on the extracellular sodium concentration ($[Na^+]_o$). At 10-30 mM $[Na^+]_o$, the magnesium efflux was faster at –10 mV than at –84 mV. Conversely, at 50-200 mM $[Na^+]_o$, the efflux of magnesium was faster at –84 mV that at –10 mV. At 75 mM $[Na^+]_o$, the activities where nearly the same at both membrane potential values. These observations suggest that the stochiometry of the Na^+/Mg^{2+} exchange changes with $[Na^+]_o$.

2. INTRODUCTION

The intracellular magnesium ($[Mg^{2+}]_i$) plays a central role in several important cellular functions, such as the regulation of enzymatic activities (1), activity of potassium channels (2) or gene transcription (3). The integrity of cellular structures such as ribosomes also requires adequate levels of magnesium (4). Thus, it is clear that the study of the ion transport mechanism that set the $[Mg^{2+}]_i$ is fundamental to understand many aspects of the cellular function.

The total cell content of magnesium is about 10 mM, most of which is bound to ATP and other cytoplasm compounds, and only about 0.5 to 1 mM is present as a free Mg^{2+}. These values are close to the magnesium concentration of the extracellular compartment (5), but far away from thermodinamical equilibrium. For example, for membrane potential of –70 mV, the expected $[Mg^{2+}]_i$ would be 188 mM. This observation indicates that the magnesium extruding mechanisms must be permanently active in order to keep $[Mg^{2+}]_i$ at the physiological levels (6).

The transport systems that regulate $[Mg^{2+}]_i$ are poorly understood. It has been proposed that Na/Mg exchangers are the major extruding magnesium systems (7). The exchange of internal Mg^{2+} for external Na^+ was first described in the giant axon of the squid Loligo forbesi (8), but subsequent studies have been reported in other tissues. Variants of the Na^+-stimulated, Mg^{2+} transport systems differ in the transport with other ionic species, the stoichiometry of the exchange, the capability of a reverse mode of operation and the sensitivity to inhibitors. As for the stoichiometry, several kinds of exchangers have been reported, either electrogenic (1Na/1Mg (7) or 3Na/1Mg (9))

or the electroneutral (2Na/1Mg (10), or 2Na,2K,2Cl/1Mg (11)).

In this work, we studied the Na-dependent Mg extruding mechanisms present in rat magfura-loaded thymocytes, in calcium-deprived medium. We attempted to identify the presence of electrogenic and electroneutral mechanisms by assessing their sensitivity to membrane potential. To do this, we loaded the cells with a high intracellular magnesium, that produced stimulation of Na-dependent magnesium extrusion, and measured the decrease in $[Mg^{2+}]_i$ induced by sodium at different membrane potential values.

3. MATERIALS AND METHODS

3.1. Materials

Male Wistar rats weighing 150-200 g were housed under adequate and constant temperature, humidity and photoperiod. Rats were fed a standard diet and were given tap water. Chemicals were of the purest analytical grade (Sigma or Aldrich, Steinheim Germany; Merck, Darmstadt Germany). The media (Na-medium) used had the following composition (in mM): 150 NaCl, 5 KCl, 10 glucose, 10 MOPS-Tris, (pH 7.4 at 20ºC). The choline medium had the same composition except that NaCl was replaced with cholineCl. In the Mg-loading process this medium was supplemented with 0.3 mM EGTA and 1 mM Na-pyruvate (se below). The osmolality of all media was adjusted to 300 ± 5 mosm kg^{-1} (Osmostat M-6020, Daichi Kagaku Co. Ltd. Kyoto Japan).

3.2. Preparation of thymocytes and mag-fura loading

Rats were anesthetized by intraperitoneal injection of 50 mg kg^{-1} sodium pentobarbital. After the disappearance of corneal and nociceptive reflexes, anaesthetized rats were laparatomized and exsanguinated by a cut on abdominal cava and aorta and the thymus was exposed by thoracotomy. Thymus glands from 1 or 2 rats were carefully and rapidly excised avoiding contamination with blood. Rats died under anesthesia during or immediately after thymus excision. Glands were washed twice at 20ºC in Na-containing medium. They were then gently and individually homogenized in 30 ml of the same medium using seven passes with a loose-fitting glass pestle (Pobel, Madrid, Spain). Each cell suspension was filtered through a thin mesh and impurities were left to sediment for five minutes. Supernatants with cells were recovered and centrifuged at 800 g and 20ºC for 5 minutes (RF Varifuge, Heraeus Sepatech, Germany). The pellet was resuspended by gently repeated pipetting in choline medium and washed twice by centrifugation under the same conditions. Small aggregates were removed and cells were finally resuspended at 10% w/v and mixed in choline medium. The resultant pool was stored at 20ºC. Three small aliquots of each cell suspension were taken for thymocryte (Heraeus Sepatech, Germany).

Thymocytes (2 % w/v in Na-medium) were loaded with 5 µM MagFura 2-AM (Molecular Probes) for 60 minutes at room temperature. Cells were centrifuged at 800 g at room temperature for 5 min, resuspended at 2% w/v in Na-medium and incubated for 30 min at 37ºC in the dark to complete desesterification of AM groups.

3.3. Mg^{2+} loading procedure

Washed, magfura 2-loaded thymocytes (2% w/v) were incubated at 20ºC for 3 min in choline medium (pH 7.4 at 20ºC) + 12 to 40 mM $MgCl_2$ (to obtain cells with different $[Mg^{2+}]_i$) + 0.3 mM EGTA + 10 µM nigericin, then 5 µM A-23187 was added and cells were additionally incubated for 5 min. Cells were centrifuged at 700 g for 5 min at room temperature and the supernatants discarded. To remove the ionophore, the pellet was washed by resuspension at 2% w/v in choline medium (without ionophore) + 1% w/v BSA and the same $MgCl_2$ (12 to 40 mM). Thymocytes were then slowly preheated to 37ºC (sharp changes in temperature were avoided to prevent aggregation) and incubated for 10 min at 37ºC. Cells were resuspended twice in the same preheated (37ºC) solution (without $MgCl_2$) and washed without incubation at 900 g and 37ºC for 5 min. Thereafter, cells were twice washed in cold (10º- 4ºC) choline medium (pH 7.4 at 6º C) to remove both BSA and external Mg^{2+}. Cell pellets were weighed, immediately resuspended at 1% w/v in cold choline medium + 0.3 mM EGTA and stored at 4ºC until use. This procedure raised the $[Mg^{2+}]_i$ from normal 0.38 ± 0.06 mM to a range between 0.6 to 8 mM free magnesium.

3.4. Measurements of intracellular magnesium

Fluorescence of magfura 2-loaded, Mg-loaded thymocytes was detected at 510 nm, alternatively exciting at 339 and 379 nm. The cells (1% w/v) were poured in quartz or polimethacrylate cuvettes, containing 2.5 mM choline medium supplemented with 0.3 mM EGTA. Cell fluorescence was measured under continuous magnetic stirring at 30ºC with a fluorescence system (Cairn Research Ltd. Faversham UK), at 30 Hz (exciting with a 5 filter-wheel excitation system) (Figures 1 and 2) or with a F-2000 Hitachi fluorometer at 2 Hz. Calibration was routinely performed at the end of the traces. The F_{min} was obtained by adding 0.1 % Triton X-100 + 2 mM EDTA + 50 mM TRIS (pH 7.4) and, subsequently, 50 mM $MgCl_2$ was added to obtain the F_{max}. Autofluorescence was measured by adding 6 mM of Mn_2Cl or Ni_2Cl, the values were similar to non-Magfura2 loaded thymocytes. The $[Mg2+]_i$ was calculated according Grynkievicz's equation using a K_d = 1.9 mM (12). Excitation spectra under F_{min}, F_{max} and autofluorescence were performed to test -AM desesterification.

3.5. Determination of the Na-dependent magnesium efflux

To determine the Na-dependent magnesium efflux, different amounts of sodium were added to the fluorescence cell containing magfura 2-loaded, Mg-loaded thymocytes incubated in choline-medium + 0.3 mM EGTA. The Na/Mg exchange activity was detected as a sodium-induced decrease in $[Mg^{2+}]_i$ in magnesium-loaded thymocytes. No difference was found when the sodium addition was either hypertonic or isotonic (traces not shown).

3.6. Determination of the effect of membrane potential on the Na-dependent magnesium efflux

To identify electroneutral or electrogenic components of the exchange, the Na-dependent magnesium efflux was assessed at two different membrane potential values. Membrane potential was held at two different

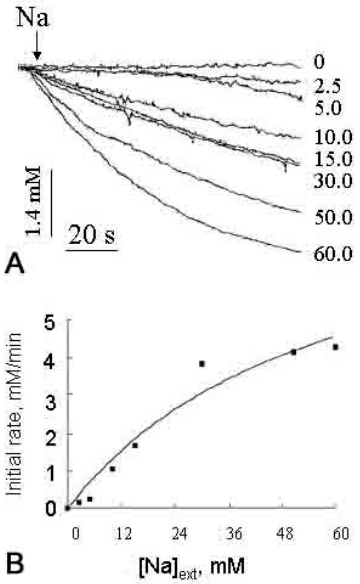

Figure 1. (A) Effect of extracellular sodium on the efflux of$[Mg^{2+}]_i$ from rat thymocytes. Magfura-2-loaded thymocytes were poured to a fluorescence cell containing 2.5 ml choline medium + 0.3 mM EGTA (0.1 % w/v), at 37ºC and under constant magnetic stirring. Initial cytosolic Mg^{2+} was 6.59 ± 0.74 mM (mean ±SEM, n= 5). The concentration of sodium (in mM) is indicated at the end of each trace. The traces represent the fluorescence values excited at 380 nm multiplied by –1. The vertical and horizontal bars are calibrations for $[Mg^{2+}]_i$ and time. (B) Plot of the initial rates as a function of the external sodium concentration. For details see the Methods section.

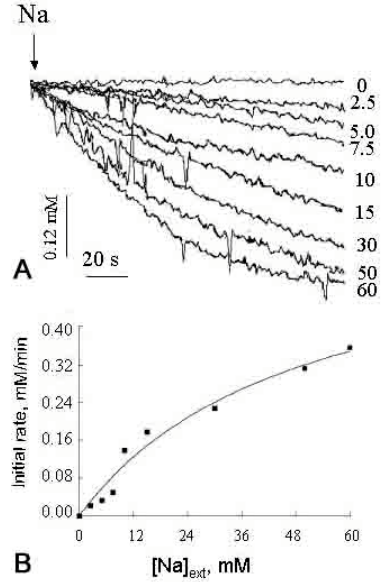

Figure 2. (A) Effect of extracellular sodium in the efflux of [Mg2+]i from rat thymocytes. Cells had been previously loaded with to a final [Mg2+]i of 0.40 ± 0.1 mM (mean□SEM, n= 5). Experimental conditions as in Figure 1. (B) Plot of the initial rates as a function of the external sodium concentration.

values by keeping the mag-fura loaded cells with the potassium ionophore valinomycin (1 μM) in either normal choline medium or in medium supplemented with 75 mM KCl. The potassium ionophore brings the membrane potential from resting, -42 mV (13), to the Nernst potential for potassium distribution (Ek= -61 mV log $([k]_{in}/[K]_{ext})$. Assuming an intracellular potassium content of 117 mM (13), the E_k values would be –84 mV in the choline medium (containing 5 mM potassium) and –10 mV in the high potassium medium (containing 80 mM potassium). The uncoupling effect of valinomycin on mitochondria and metabolism seemed no to affect the Na/Mg exchange activity reported here since when triggered with 75 mM Na, the activity was similar both in the presence and the absence of valinomycin in normal medium (see Results).

4. RESULTS

Figures 1 and 2 show the effects the of increasing concentration of extracellular sodium on the time course of $[Mg^{2+}]_i$ decrease in magfura 2-loaded thymocytes loaded with magnesium at two different levels, 6.6 mM and 0.6 mM. In the cells loaded with the higher magnesium concentration (Figure 1), addition of sodium produced a decrease in $[Mg^{2+}]_i$ that depended on the sodium concentration, the higher the sodium concentration, the higher the $[Mg^{2+}]_i$ decrease. The apparent K_m (for external Na) and V_{max} values were of 59 mM and 9 mmol/min, respectively (figure 1B). When the cells were loaded with the lowest magnesium concentration ($[Mg^{2+}]_i$ = 0.6 mM) the Na-stimulated $[Mg^{2+}]_i$ decrease was much slower (figure 2A and B) and the apparent K_m (for Na) and V_{max} values decreased to 49 mM and 0.63 mmol/min, respectively. In intact thymocytes, that is, no loaded with magnesium, $[Mg^{2+}]_i$ was 0.38 ± 0.06 mM (n = 5), and no effect of external sodium (up to 200 mM) was observed (trace not shown).

To investigate whether the Na-stimulated magnesium efflux is electrogenic or electroneutral, we studied the activity at two different membrane potential values, in both, high and low magnesium-loaded thymocytes. Figure 3 shows the effect of 10, 30, 75, 150 and 200 mM hypertonic sodium on the time course of $[Mg^{2+}]_i$ decrease, both at –84 and –10 mV in thymocytes containing $[Mg^{2+}]_i$ = 8 mM. At the lowest sodium concentrations, 10 and 30 mM, the Na-stimulated $[Mg^{2+}]_i$ decrease were 2- fold faster at –10 mV than at –84 mV (Figure 3 and 5A). Conversely, at higher sodium concentrations, 50 and 200 mM, the exit of magnesium was 1.4-3 folds faster at –84 mV than at –10 mV. At 75 mM external sodium, the activity was almost the same at –84 and at at –10 mV. Furthermore, at this concentration the exchange activity was similar both in the presence and the absence of valinomycin in normal medium (not shown) indicating that the exchange was unaffected by the uncoupling effect of the ionophore on mitochondria. Interestingly, at [Na] > 75 mM, even though the initial rates were higher at –84 than at –10 mV, the activity at later times were rather reversed, that is, the activity at –90 mV tended to be slower than at –13 mV.

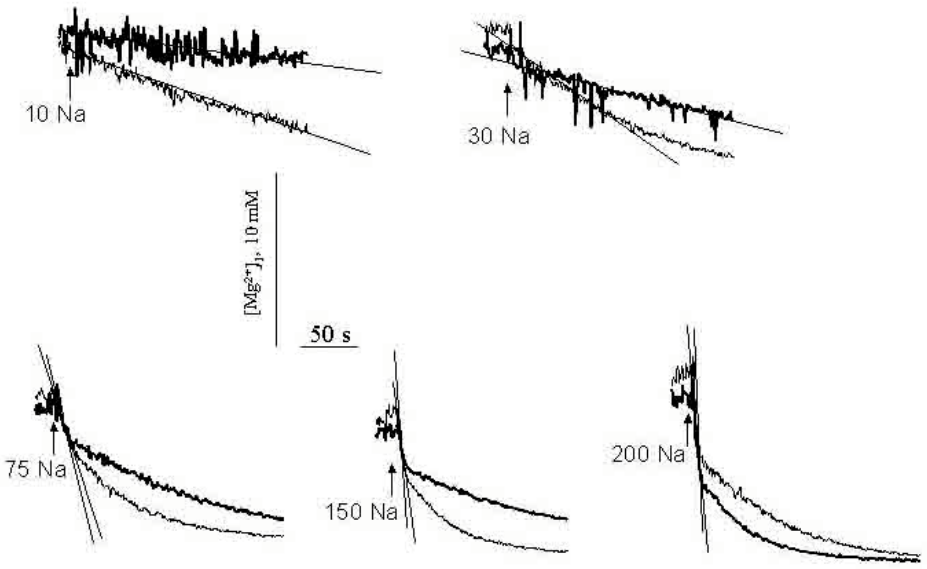

Figure 3. Effects of membrane potential on the Na^+-stimulated $[Mg^{2+}]_i$ decrease. Cells were loaded to a $[Mg^{2+}]_i$ of 8mM. The cells were then poured in the fluorescence cell containing 2.5 ml choline medium + 0.3 mM EGTA. Valinomycin (1 μM) was added to the medium at the beginning of the records (estimated membrane potential, -84 mV). In the another set of experiments, the membrane potential was brought to –10 mV by supplementing the incubation medium with 75 mM KCl. The amount of sodium added (mM) is indicated by the arrows. The traces drawn in boldface are at –84 mV. The vertical and horizontal bars are calibrations for $[Mg^{2+}]_i$ and time. The slopes representing the initial rates are drawn on the traces.

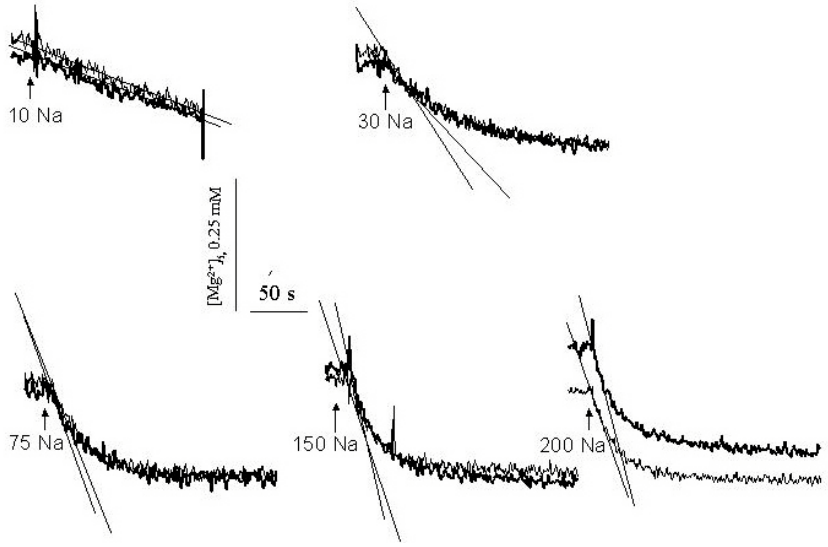

Figure 4. Effect of membrane potential on the Na^+-stimulated $[Mg^{2+}]_i$ efflux. Cells were loaded to a $[Mg^{2+}]_i$ of 0.6 mM. The membrane potential was either –84 mV (drawn boldface) or–13 mV. The rest of the experimental conditions are described in figure 3.

Similar results where obtained with the cells loaded to the lowest $[Mg^{2+}]_i$ (0.6 mM). As shown in figures 4 and 5B, when the magnesium extrusion was stimulated at >150 mM Na the activity tended to be 25-50 % higher at –84 mV than at –10 mV. At 30 mM sodium the effect was opposite, slightly higher at –10 than at –84 mV. In contrast with high $[Mg^{2+}]_i$ thymocytes, in low $[Mg^{2+}]_i$ thymocytes the activity at later times tended to reach similar values at both membrane potential.

5. DISCUSION

In this paper we provide evidence indicating that rat thymocytes possess a magnesium extruding system(s) that is (are) driven by extracellular sodium, which can be detected by measuring the changes of fluorescence in magfura-2 loaded thymocytes incubated in choline medium + 0.3 mM EGTA. Another important requirement is that the intracellular content of free magnesium must reach a

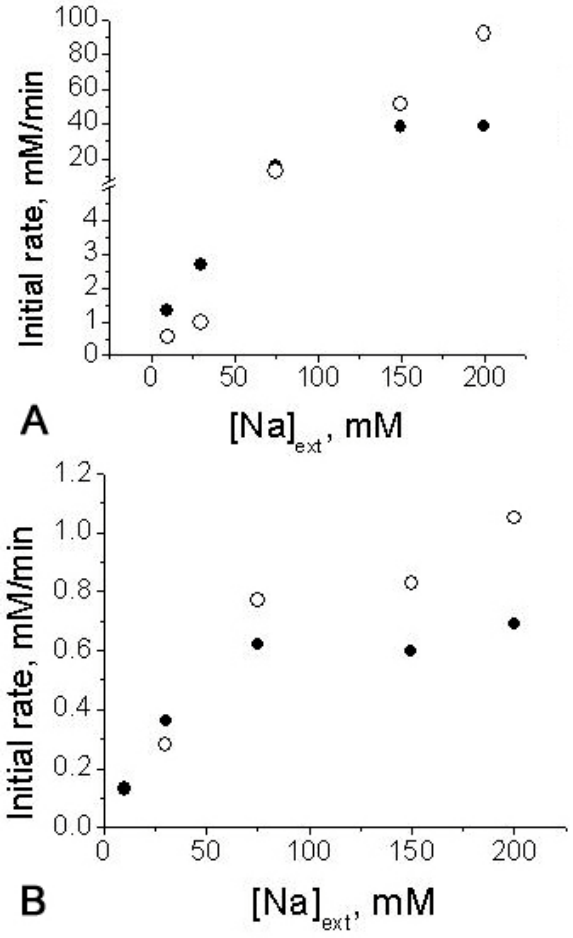

Figure 5. Comparison of the initial rates (slopes) of Mg^{2+} extrusion as a function of external sodium at –84 mV (open circles) and at –13 mV (closed circles). A, curve obtained from traces from figure 3, with high magnesium-loaded thymocytes ($[Mg^{2+}]_i$ = 8 mM); B, curve form obtained from traces from figure 4, with low magnesium loaded thymocytes ($[Mg^{2+}]_i$ = 0.6 mM).

sufficiently high value to result in a magfura-2 fluorescence decay through magnesium efflux. Interestingly, Vmax increased markedly when the intracellular magnesium content was increased indicating that, beside the higher efflux promoted by the chemical gradient, the functional properties of the transport system are modified by $[Mg^{2+}]_i$. This phenomenon indicates that the stimulating effect of sodium on magnesium efflux on the external side observed here is strongly stimulated by magnesium present at the intracellular side.

Our results are consistent with the notion that the Na-dependent $[Mg^{2+}]_i$ decrease is related to activation of one or different Na/Mg exchanger(s). In other systems, these exchangers may operate with different stoichiometries, an electrogenic 1Na/1Mg found in Retzius neurones and glial cells of the leech Hirudo medicinalis (6) and the 3Na/1Mg exchangers found in human red blood cells (8) and the electroneutral 2Na/1Mg exchanger found in chicken red blood cells (9). The fact that the initial rate of the putative Na/Mg exchanger was faster at –10 mV than at –84 mV when the external sodium concentration was low (10-30 mM) suggests that the main contributor of the Na/Mg exchange was the electrogenic 1Na/1Mg exchange, that moves a net positive charge out of the cell in every exchange cycle. Conversely, the higher activity of the Na/Mg exchange at –84 mV as compared with –10 mV when the external sodium was high (150-200 mM) suggests that activation of a 3Na/1Mg electrogenic system, moving net one positive charge into the cell during the cycle, is mainly involved at high external sodium concentration. The Km for external sodium for this exchanger would be expected to be > 75 mM. At 75 mM external sodium the membrane potential had little effect on the exchange activity. This suggests that at this concentration, the 1Na/1Mg and the 3Na/1Mg exchangers contribute similarly to the efflux of magnesium. Besides, the electroneutral 2Na/1Mg exchanger, that has been reported in Mg-loaded rat thymocytes (14), might be activated at this sodium concentration.

These hypotheses are based exclusively on the analysis of the effect of membrane potential on initial rates, however, unlike what was observed at initial rates, the time course of $[Mg^{2+}]_i$ decrease induced by [Na] > 75 mM at later times, in high loaded magnesium cells, was lower at –84 than at –10 mV. This was not observed in cells loaded with a low magnesium content, suggesting that the effect of intracellular sodium, that should be remarkably enhanced during the activity induced by > 75 mM [Na] in high magnesium loaded thymocytes, could down regulate the activity. Since this down regulation would occur at –84 mV, the 3Na/1Mg might be preferentially affected. Further research is required to determine if internal sodium modulates the 3Na/1Mg exchanger.

In summary, in this paper we provide evidence, based exclusively in the analysis of cells loaded with mag-fura, indicating that rat thymocytes are endowed with Na/Mg exchangers. The effect of membrane potential on the activity suggests that exchangers with different stoichiometries can operate in the cell, the contribution of which depends on the external sodium.

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