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ORIGINAL PAPER

Modulation of Mg^{2+} influx and cytoplasmic free Mg^{2+} concentration in rat ventricular myocytes

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Abstract

To examine whether TRPM7, a member of the melastatin family of transient receptor potential channels, is a physiological pathway for Mg^{2+} entry in mammalian cells, we studied the effect of TRPM7 regulators on cytoplasmic free Mg^{2+} concentration ($[Mg^{2+}]_i$) of rat ventricular myocytes. Acutely isolated single cells were AM-loaded with the fluorescent indicator furaptra, and $[Mg^{2+}]_i$ was estimated at 25 °C. After $[Mg^{2+}]_i$ was lowered by soaking the cells with a high-K^+ and Mg^{2+}-Ca^{2+}-free solution, $[Mg^{2+}]_i$ was recovered by extracellular perfusion of Ca^{2+}-free Tyrode's solution that contained 1 mM Mg^{2+}. The initial rate of increase in $[Mg^{2+}]_i$ was analyzed as the Mg^{2+} influx rate. The Mg^{2+} influx rate was increased by the TRPM7 activator, naltriben (2–50 μM), in a concentration-dependent manner with a half maximal effective concentration (EC_{50}) of 24 μM. This EC_{50} value is similar to that reported for the activation of recombinant TRPM7 overexpressed in HEK293 cells. Naltriben (50 μM) caused little change in basal $[Mg^{2+}]_i$ (~0.9 mM) in Ca^{2+}-free Tyrode's solution, but significantly raised $[Mg^{2+}]_i$ to 1.31 ± 0.03 mM in 94 min after the removal of extracellular Na^+. Re-introduction of extracellular Na^+ lowered $[Mg^{2+}]_i$ back to the basal level even in the presence of naltriben. Application of 10 μM NS8593, an inhibitor of TRPM7, significantly lowered $[Mg^{2+}]_i$ to 0.72 ± 0.03 mM in 50-60 min independent of extracellular Na^+. The results suggest that Mg^{2+} entry through TRPM7 significantly contributes to physiological Mg^{2+} homeostasis in mammalian heart cells.

Keywords Magnesium influx · TRPM7 · Naltriben · NS8593 · Rat ventricular myocyte

Introduction

Cytoplasmic free Mg^{2+} concentration ($[Mg^{2+}]_i$) is estimated at or slightly below 1 mM in many types of cells, which is much lower than that expected from passive distribution of the ion across the cell membrane. However, molecular mechanisms responsible for intracellular Mg^{2+} homeostasis are largely unknown. Because electrochemical potential gradient drives Mg^{2+} inward, any Mg^{2+}-permeable channels could be involved in passive Mg^{2+} entry. Among transient receptor potential (TRP) cation channel families, TRPM7 (melastatin subfamily, member 7) has attracted particular attention as a possible Mg^{2+} entry pathway, because TRPM7 is (1) permeable to divalent cations including Mg^{2+} [1], (2) regulated by $[Mg^{2+}]_i$ [2], and (3) ubiquitously expressed [3–5]. However, it is still unclear whether TRPM7 plays an important role in physiological Mg^{2+} entry into native mammalian cells.

The physiological Mg^{2+} channels are challenging to identify due to the low permeation rate of Mg^{2+} under physiological conditions, which make it very difficult to reliably detect Mg^{2+} current via electrophysiology. We previously devised a method to quantitatively monitor Mg^{2+} entry into ventricular myocytes acutely isolated from rat hearts, and reported that the rate of Mg^{2+} influx was reduced by known inhibitors of TRPM7 [6–8] in parallel with its channel activities [9].

Hofmann et al. [10], who recently reported 20 small-molecule activators of TRPM7, showed that one of the compounds, naltriben, is a positive gate modulator of TRPM7. In the present study, we studied the effect of naltriben on the Mg^{2+} influx rate in rat ventricular myocytes. Modulation of the basal $[Mg^{2+}]_i$ by TRPM7 regulators, naltriben (an activator) and NS8593 (an inhibitor), were also tested. Some of the results have been reported in abstract form [11].

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Materials and methods

General

All experiments were undertaken with the approval of the Institutional Animal Care and Use Committee (IACUC) of Tokyo Medical University (Permit No.S-27029).

Fluorescence signals from single myocytes were measured as previously described [12]. Cardiac ventricular myocytes were enzymatically dissociated from hearts of male Wister rats (9–15 weeks old) [13]. Single myocytes were placed in a chamber on the stage of an inverted microscope (TE300; Nikon, Tokyo) and then superfused with normal Tyrode's solution containing (mM) 135 NaCl, 5.4 KCl, 1.0 $CaCl_2$, 1.0 $MgCl_2$, 0.33 NaH_2PO_4, 5.0 glucose and 10 HEPES (pH 7.40 at 25 °C by NaOH).

Measurements and analyses of furaptra signals

Single ventricular myocytes were loaded with 5 μM furaptra AM (mag-fura-2 AM) by incubation in normal Tyrode's solution for 14 min at room temperature. The acetoxy methyl (AM) ester was washed out with Ca^{2+}-free Tyrode's solution that contained 0.1 mM K_2EGTA in place of 1.0 mM $CaCl_2$ of normal Tyrode's solution for at least 10 min. Subsequent fluorescence measurements were carried out at 25 °C under Ca^{2+}-free conditions to minimize possible cell damage and interference in the furaptra fluorescence caused by Ca^{2+} overloading of the cells.

The intracellular furaptra was alternately excited with 350- and 382-nm light beams at 10-ms intervals, and the fluorescence at 500 nm (25-nm bandwidth) was detected from the entire volume of the single cell under study. At each excitation wavelength, the background fluorescence, measured for each cell before indicator loading, was subtracted from the total fluorescence measured after indicator loading to yield indicator fluorescence intensity. The ratio of furaptra fluorescence intensities excited at 382 and 350 nm [$R = F(382)/F(350)$] was converted to $[Mg^{2+}]_i$ according to the equation:

$$\left[Mg^{2+}\right]_i = K_D \cdot \frac{R - R_{min}}{R_{max} - R} \quad (1)$$

where K_D is the dissociation constant, and R_{min} and R_{max} are R values at zero $[Mg^{2+}]$ and saturating $[Mg^{2+}]$, respectively. We used the parameter values previously estimated in rat ventricular myocytes ($K_D = 5.30$ mM, $R_{max} = 0.223$), and those newly estimated in the present study ($R_{min} = 0.967$). Note that the R_{min} value was very similar to that previously reported (0.969) [14].

Depletion of intracellular Mg^{2+} and analysis of Mg^{2+} influx rate

The experimental protocol used for depletion and recovery of $[Mg^{2+}]_i$ has been previously described [9]. In brief, the cells were depleted of Mg^{2+} by incubation (for 20 min at 35 °C) in the Mg^{2+}-depleting solution [(mM): 140 KCl, 0.33 NaH_2PO_4, 5.0 glucose and 10 HEPES, 0.1 K_2EGTA (pH 7.40 at 25 °C by KOH)], which caused a decrease in $[Mg^{2+}]_i$ from the basal level (~0.9 mM) to 0.2–0.5 mM.

When the Mg^{2+}-depleted myocytes (0.2–0.5 mM $[Mg^{2+}]_i$) were superfused with Ca^{2+}-free Tyrode's solution with or without an activator of TRPM7 (naltriben) at 25 °C, $[Mg^{2+}]_i$ started to rise and reached a plateau near the initial basal level; the $[Mg^{2+}]_i$ recovery was followed at ~2-min intervals for 150–180 min. We found that the time course of the recovery could be well fitted by a two-phase exponential function (a sum of two exponential functions), rather than a single exponential function used in the previous study [9], particularly when the $[Mg^{2+}]_i$ recovery showed an overshoot (e.g., Fig. 1c).

With time (t),

$$\left[Mg^{2+}\right]_i(t) = A_1 \cdot \exp(-t/\tau_1) + A_2 \cdot \exp(-t/\tau_2) + [Mg^{2+}]_i(t = \infty) \quad (2)$$

where A_1 (<0) or A_2 (>0) is a constant. τ_1 and τ_2 are time constants for, respectively, the rise and the decay of $[Mg^{2+}]_i$. Because the $[Mg^{2+}]_i$ recovery is likely caused by the net influx of Mg^{2+}, the first derivative of the recovery function (Eq. 2) is thought to reflect the rate of net Mg^{2+} influx,

$$d\left[Mg^{2+}\right]_i(t)/dt = (-A_1/\tau_1) \cdot \exp(-t/\tau_1) + (-A_2/\tau_2) \cdot \exp(-t/\tau_2) \quad (3)$$

We used the value of $d[Mg^{2+}]_i(t)/dt$ at time 0, $-A_1/\tau_1 - A_2/\tau_2$, as an index of the initial rate of Mg^{2+} influx.

Solutions and chemicals

When extracellular Na^+ was removed, 135 mM NaCl of Ca^{2+}-free Tyrode's solution was substituted with 135 mM *N*-methyl-D-glucamine-Cl (NMDG-Tyrode's solution). Naltriben methanesulfonate hydrate (naltriben) and NS8593 hydrochloride (NS8593) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Furaptra (tetrapotassium salt of mag-fura-2), furaptra AM (mag-fura-2 AM) were purchased from Invitrogen (Life Technologies, Carlsbad, CA, USA). Water-insoluble compounds were dissolved from their concentrated stock solutions in DMSO. The final concentration of the solvent was < 0.1% during fluorescence measurements, which did not affect the $[Mg^{2+}]_i$ measurements.

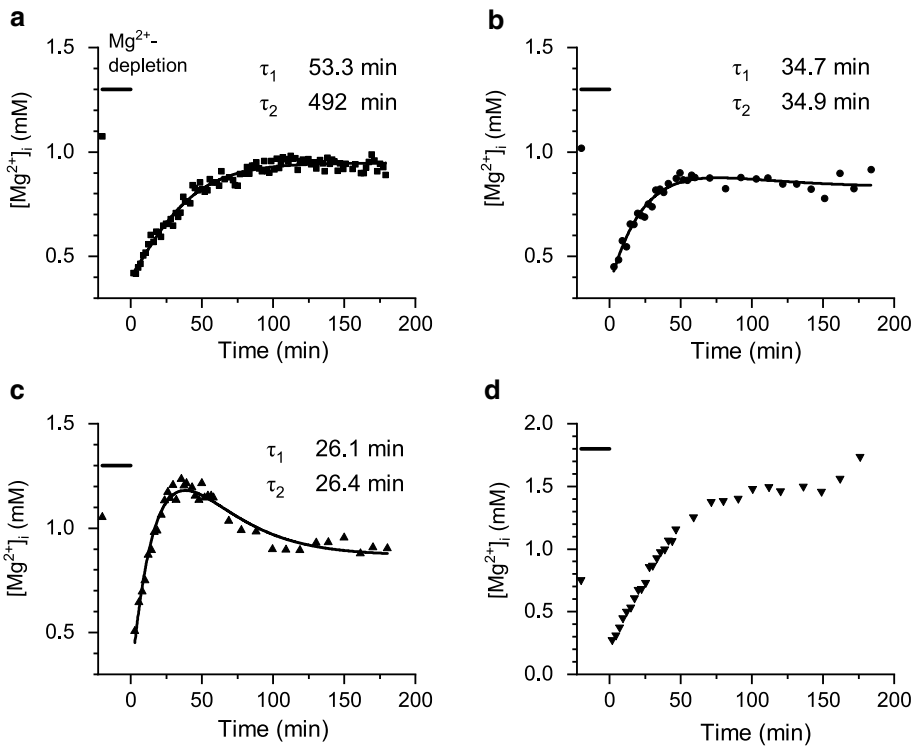

Fig. 1 Examples of $[Mg^{2+}]_i$ recovery with or without naltriben. Each symbol shows the data obtained from individual cells. The *black bar* on the left-upper side in each panel shows the period of Mg^{2+} depletion. The value of $[Mg^{2+}]_i$ at time -20 (min) indicates the basal $[Mg^{2+}]_i$ before depletion. After time 0, cells were perfused with the following: **a** Ca^{2+}-free Tyrode's solution without naltriben, **b** naltriben at 5 μM, **c** 50 μM, and **d** 100 μM. Each *solid line* in **a**–**c** was drawn according to a two-phase exponential fitting of the data set (see "Materials and methods") with time constants (min) shown in each panel

Data analysis

Nonlinear least-squares curve fitting was performed with the program Origin (Ver. 9.1, Origin Lab, Northampton, MA, USA). Statistical values are expressed as the mean ± SE. Differences between groups were analyzed by Student's two-tailed *t* test with the significance level set at $p < 0.05$.

Results

Effects of naltriben on the Mg^{2+} influx

As shown in Fig. 1a, the lowered $[Mg^{2+}]_i$ was recovered to the steady level near basal $[Mg^{2+}]_i$ by perfusion of the Ca^{2+}-free Tyrode's solution that contained 1 mM Mg^{2+}. The administration of 5 μM naltriben increased the speed of the $[Mg^{2+}]_i$ recovery (Fig. 1b). The higher concentration of naltriben (50 μM) further steepened the slope of the $[Mg^{2+}]_i$ recovery. In six out of nine cells treated with 50 μM naltriben, $[Mg^{2+}]_i$ rose transiently above the steady level (Fig. 1c). This overshoot phenomenon could be similarly observed when the Mg^{2+} influx was facilitated by high extracellular $[Mg^{2+}]$ (not shown). It should be noted that the entire time course of the $[Mg^{2+}]_i$ recovery, including the overshoot, was well fitted by the two-phase exponential function (see Methods).

Interestingly however, with 100 μM naltriben we unexpectedly found that the $[Mg^{2+}]_i$ recovery was delayed, and, in most cells, $[Mg^{2+}]_i$ continuously rose to ~ 2 mM or higher without reaching the steady state (Fig. 1d). The time course of the $[Mg^{2+}]_i$ change could be fitted only partially by the two-phase exponential function. Because this rise of $[Mg^{2+}]_i$ could be due to drug-induced toxicity by the high concentration of naltriben, as generally observed in many types of cell damage [15], the results with 100 μM naltriben were not included in the following analyses.

Table 1 summarizes the results of this type of experiment. Among groups of cells treated with 0 (control) – 50 μM naltriben, the basal $[Mg^{2+}]_i$ and the initial $[Mg^{2+}]_i$ were not significantly different, indicating similar initial conditions prior to naltriben treatment. The Mg^{2+} influx rate was significantly greater in the presence of naltriben at 5 μM or higher.

Figure 2 shows the concentration dependence of naltriben on the rate of Mg^{2+} influx. Data obtained at 0-50 μM naltriben were least-squares fitted with the Hill-type curve,

$$\text{Initial } d[Mg^{2+}]_i/dt = \text{min} + \frac{(\text{max} - \text{min})[\text{naltriben}]^N}{EC_{50}^N + [\text{naltriben}]^N} \quad (4)$$

where min is the rate in the absence of naltriben (0.20 μM/s), max is the maximum rate (0.71 μM/s), *N* is the Hill coefficient (0.45), and EC_{50} is the half-maximal effective concentration (24 μM).

Table 1 Summary of the initial rate of Mg^{2+} influx obtained at various concentrations of naltriben

Naltriben (μM)	0	2	5	10	20	50
Basal $[Mg^{2+}]_i$ (mM)	0.92 ± 0.04	1.02 ± 0.026	0.97 ± 0.02	0.98 ± 0.04	0.94 ± 0.02	0.95 ± 0.04
Initial $[Mg^{2+}]_i$ (mM)	0.37 ± 0.01	0.42 ± 0.02	0.34 ± 0.04	0.32 ± 0.04	0.32 ± 0.05	0.36 ± 0.05
Mg^{2+} influx rate (μM/s)	0.22 ± 0.03	0.30 ± 0.07	0.41 ± 0.05**	0.41 ± 0.07*	0.36 ± 0.06*	0.52 ± 0.10**
Number	11	7	10	7	6	9

Basal $[Mg^{2+}]_i$: $[Mg^{2+}]_i$ measured just before Mg^{2+} depletion. Initial $[Mg^{2+}]_i$: $[Mg^{2+}]_i$ at the beginning of Mg^{2+} recovery (defined as $[Mg^{2+}]_i$ at time 0 on the fitted curve). Each data shows mean ± SE from the number (listed in the bottom row) of cells

Statistical differences show $^{**}p < 0.01$, $^{*}\ 0.01 \le p < 0.05$ (vs. 0 μM naltriben)

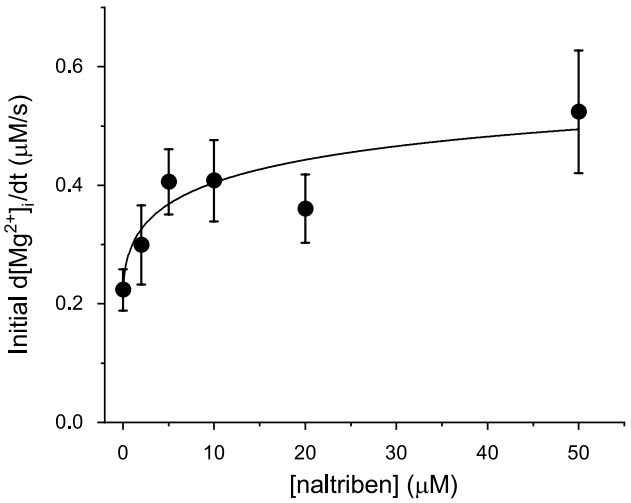

Fig. 2 Concentration-dependent activation of the Mg^{2+} influx by naltriben. The initial rates of rise in $[Mg^{2+}]_i$, as estimated and shown in Fig. 1, were plotted as a function of naltriben concentration (0, 2, 5, 10, 20, and 50 μM). Each symbol represents mean ± SE of the rates at each concentration (see Table 1). A *solid line* was drawn by least-squares fit of the data with the Hill-type curve as described in "Results"

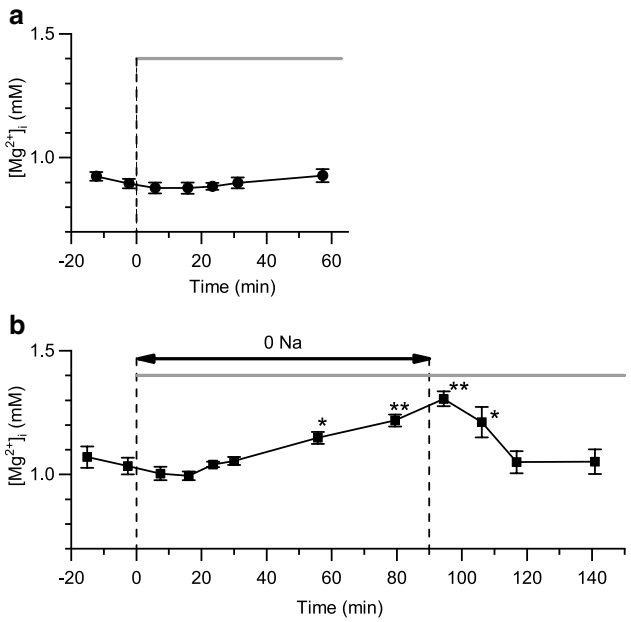

Fig. 3 Effects of naltriben on the basal $[Mg^{2+}]_i$. Each symbol represents the mean ± SE of the data obtained from 7 (**a**) or 6 (**b**) cells. Cells were perfused with Ca^{2+}-free Tyrode's solution except during the period of perfusion with NMDG Tyrode's solution (0Na as indicated by the line with *arrows* in **b**). *Gray bars* on the top of each panel indicate the period of administration of 50 μM naltriben. $^{*}0.01 \le p < 0.05$, $^{**}p < 0.01$ versus the average of $[Mg^{2+}]_i$ just before the drug administration (at time − 2 min)

Effects of naltriben on the basal $[Mg^{2+}]_i$

The basal levels of $[Mg^{2+}]_i$ of rat ventricular myocytes were stable in the Ca^{2+}-free Tyrode's solution. We previously reported that removal of extracellular Na^+ did not cause a significant change in $[Mg^{2+}]_i$ for 30 min, even with 10 mM extracellular Mg^{2+} [16]. The average value of the basal $[Mg^{2+}]_i$ just before administration of the drug (at time − 2 min in Figs. 3 and 4a, b) was 0.90 ± 0.03 mM. Naltriben (50 μM) caused little change in $[Mg^{2+}]_i$ within 57 min in Ca^{2+}-free Tyrode's solution that contained 140 mM Na^+ (Fig. 3a). However, when 50 μM naltriben was applied in NMDG-Tyrode's solution that contained only 0.3 mM Na^+ (0 Na), $[Mg^{2+}]_i$ was raised significantly in 56 min, and was further elevated in 80 min (Fig. 3b). Upon reintroduction of extracellular Na^+ by superfusion with Ca^{2+}-free Tyrode's solution, $[Mg^{2+}]_i$ rapidly fell and the steady level reached near the basal level (Fig. 3b).

Effects of NS8593 on the basal $[Mg^{2+}]_i$

Because the activator of TRPM7 was found to raise the basal $[Mg^{2+}]_i$ in the absence of extracellular Na^+, we studied the effects of NS8593, an inhibitor of TRPM7 [8], on the basal $[Mg^{2+}]_i$. It has been shown that NS8593 specifically targets TRPM7 among many TRP channels overexpressed in HEK293 cells [8]. In rat ventricular myocytes, we previously confirmed that NS8593 strongly

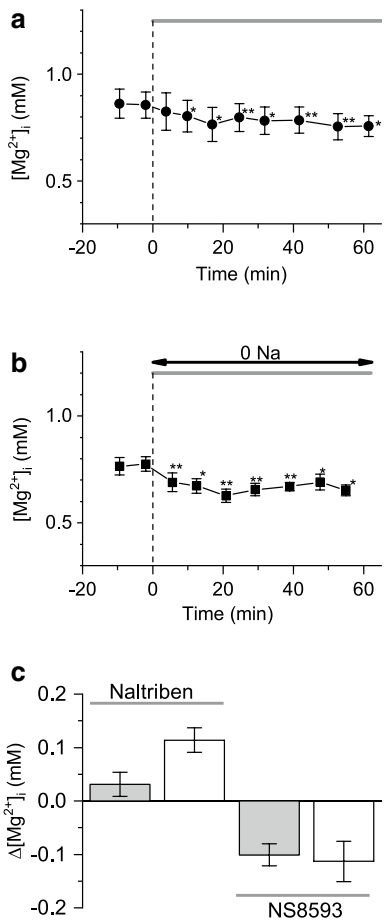

Fig. 4 Effects of NS8593 on the basal $[Mg^{2+}]_i$. **a**, **b** Each symbol represents mean ± SE of $[Mg^{2+}]_i$ obtained from 5 cells. Cells were perfused with Ca^{2+}-free Tyrode's solution except during the period of perfusion with NMDG Tyrode's solution indicated by the line with arrows in **b** (0Na). *Gray bars* indicate the period of administration of 10 μM NS8593. *$0.01 \leq p < 0.05$, **$p < 0.01$ versus the average of $[Mg^{2+}]_i$ just before the drug administration (at time − 2 min). **c** Columns summarize the change of $[Mg^{2+}]_i$ ($\Delta[Mg^{2+}]_i$) 50–60 min after application of naltriben (Fig. 3a, b) or NS8593 (**a**, **b**). *Gray* or *white columns* show mean ± SE of the data obtained, respectively, with or without extracellular Na^+

inhibited Mg^{2+} influx (measured as shown in Fig. 1 of the present study) with half-maximal inhibitory concentration of 2 μM [9].

After the application of NS8593 (10 μM), $[Mg^{2+}]_i$ was significantly decreased by ~ 0.1 mM in 50–60 min either in the presence of 140 mM extracellular Na^+ or in its essential absence (Fig. 4a, b). The effects of naltriben and NS8593 on the basal $[Mg^{2+}]_i$ are summarized in Fig. 4c. Naltriben increased $[Mg^{2+}]_i$ (positive $\Delta[Mg^{2+}]_i$) only in the absence of extracellular Na^+, and NS8593 decreased $[Mg^{2+}]_i$ (negative $\Delta[Mg^{2+}]_i$) independent of extracellular Na^+.

Discussion

In search of activators of TRPM7, Hofmann et al. [10] screened 1280 substances, and selected 20 compounds based on $[Ca^{2+}]_i$ assay using aequorin bioluminescence. HEK 293 cells expressing mouse TRPM7 (and aequorin) were treated with each activator, and the elevation of $[Ca^{2+}]_i$, caused by Ca^{2+} influx through TRPM7, was monitored. They found that naltriben, a δ opioid antagonist, activated Ca^{2+} entry through TRPM7 with an EC_{50} value of 21 μM, and it was selective over other TRP channels [10].

In the present study, we utilized naltriben as a tool to modulate TRPM7 activity in rat ventricular myocytes, and found that activation of the channel caused an increase in the rate of Mg^{2+} entry with an EC_{50} value (24 μM) very similar to that reported for Ca^{2+} entry in HEK293 cells (see above). The results strongly suggest that endogenous TRPM7 of native heart cells can mediate Mg^{2+} influx and elevate $[Mg^{2+}]_i$.

Under basal conditions, the application of naltriben (50 μM) induced a rise in $[Mg^{2+}]_i$ only in the absence of extracellular Na^+ (Fig. 3). We have previously shown that a slight elevation of $[Mg^{2+}]_i$ above the basal level activates Mg^{2+} efflux, of which activity critically depends upon extracellular Na^+ with half-maximal activation at 55 mM (i.e., the putative Na^+/Mg^{2+} exchange) [17]. It follows that, in the presence of extracellular 140 mM Na^+, Mg^{2+} entry via TRPM7 could be counterbalanced with Mg^{2+} extrusion via the Na^+/Mg^{2+} exchange, causing little change in $[Mg^{2+}]_i$ (Fig. 3a). Inhibition of the Mg^{2+} efflux by removal of extracellular Na^+ should uncover the Mg^{2+} entry, as revealed by the rise in $[Mg^{2+}]_i$. The re-addition of extracellular Na^+ is thought to 'turn on' the Na^+/Mg^{2+} exchange that would subsequently return $[Mg^{2+}]_i$ back to the basal level (Fig. 3b).

Because naltriben may affect Mg^{2+} channels/transporters other than TRPM7, we also used NS8593 to modulate basal $[Mg^{2+}]_i$. When TRPM7 was inhibited by NS8593, $[Mg^{2+}]_i$ fell below the basal level, independent of extracellular Na^+ (Fig. 4), suggesting that Na^+-independent Mg^{2+} efflux might come into play below the basal $[Mg^{2+}]_i$. Thus, the present results suggest that, even at the basal level, a dynamic equilibrium exists between the Mg^{2+} entry via TRPM7 and Mg^{2+} extrusion via Na^+-dependent and Na^+-independent pathways.

Although the possible cell toxic effects of naltriben have not been previously reported [10], we unexpectedly found that naltriben showed signs of cell toxicity at a high concentration, 100 μM (Fig. 1d). The difference could be due to the period of drug application. Hoffmann et al. [10] used $[Ca^{2+}]_i$ assay that lasted within a few minutes,

whereas the present study monitored $[Mg^{2+}]_i$ for tens of minutes or longer in the presence of the drug. Although naltriben appears to be a useful tool to modulate TRPM7 activities, care should be taken when applied at high concentrations for prolonged periods.

With facilitated Mg^{2+} influx, $[Mg^{2+}]_i$ recovery from depletion was often biphasic (Fig. 1c), which could be well fitted by a sum of two exponential functions (Eq. 2). This function was used as a description of the time course of Mg^{2+} influx to facilitate comparison of the data at different drug concentrations. Although it is tempting to speculate that two exponents represent activities of Mg^{2+} influx and efflux, assessment of their physical significance should await further studies.

In summary, we conclude that activation of TRPM7 increases the rate of Mg^{2+} influx, which raises basal $[Mg^{2+}]_i$ when Mg^{2+} efflux is inhibited. To our knowledge, this is the first report to show that TRPM7, endogenously expressed in native mammalian cells, functions as a part of Mg^{2+} homeostasis under near physiological conditions.

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Authors' contributions All authors conceived and designed the study. MT performed the experiments, analyzed data, and wrote the initial draft of the manuscript. MK contributed to analysis and interpretation of data, and wrote the manuscript. HI contributed to data interpretation, and critically reviewed the manuscript. All authors approved the final version of the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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