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ABSTRACT

The widely expressed, nonselective cation channel TRPM7 has recently been shown to permit entry of trace metals, at least when the metal ion is applied at high concentrations in voltage clamp experiments. These results lead to the suggestion that TRPM7 channels may be involved in the homeostatic regulation of trace metal content in cells. It is not clear, however, that extrapolation from high-dose behavior is warranted or that trace metals could effectively compete with the more abundant Ca^{2+} and Mg^{2+} ions in traversing the channel. In order to accept the proposal that TRPM7 is involved in the physiological regulation of intracellular trace metals, it would be necessary to demonstrate trace metal influx at concentrations similar to what would be encountered in an organism and in the presence of competing ions.

Using fluorescent imaging with indicator dyes and whole-cell patch clamp recordings, we explored the entry of a selected trace metal ion, Mn^{2+}, through TRPM7 channels. We studied the rate of Mn^{2+} influx into cells when Mn^{2+} is applied at concentrations approaching the estimated physiological range in the presence of physiologically appropriate levels of Ca^{2+} and Mg^{2+}. We also looked at Mn^{2+} influx in the presence of either external Ca^{2+} or Mg^{2+}. Our results show that imaging methods can resolve the entry of Mn^{2+} over a range far below what can be measured by voltage clamp methods, in the presence of competing ions. Furthermore, we show that Ca^{2+} and Mg^{2+} have differing effects on Mn^{2+} entry, with Ca^{2+} causing a particularly strong block in the absence of Mg^{2+}. We also studied the effects of Mn^{2+} on TRPM7 channel function. Our results show that internal Mn^{2+} either inhibits or augments TRPM7 currents depending on Mn^{2+} concentration. Whether current size is increased or decreased, the effects of internal Mn^{2+} show some voltage dependence, being more pronounced at depolarized voltages.

The present studies contribute to our understanding of ion-ion interactions likely to modulate trace metal entry by this pathway.

METHODS

Cell Culture

We used tetracycline (TET) - inducible murine TRPM7 (mTRPM7) heterologously expressed in HEK-293 cells (generously provided by A. Scharenberg, University of Washington). These were grown at 37°C in DMEM medium supplemented with 10% FBS, 5 µg/ml blasticidin and 0.4 mg/ml zeocin in the presence of 5% CO_2 and passaged twice a week. At the time of passage, cells were plated onto glass cover slips coated with high molecular weight (>10,000 MW) poly-lysine to enhance adhesion during imaging experiments. Plated cells were divided into two groups. One was treated with 1µg/ml TET to induce mTRPM7 expression while the other group of sister cells served as a control group. Experiments were done 24-48 hrs after induction.

By using whole-cell patch clamp measurements, we verified that more than 90% of cells respond to TET treatment by up-regulating mTRPM7 expression. These cells generally acquire a rounded morphology 24 to 48 hrs later. Native HEK-293 cells and transfected cells not exposed to TET commonly have elongated processes. We took advantage of this observation to identify induced cells expressing mTRPM7 during our imaging experiments, when there was no way to measure whole-cell currents. This helped to limit data variability.

Fluorescence Imaging

The indicator dye fluo-3 (Molecular Probes) was used to monitor **Mn^{2+}** influx. Measured in cell-free systems, **Kd** for **Mn^{2+}** binding to fluo-3 is approximately **6.4 nM** while the **Kd** for **Ca^{2+}** is around **400 nM** (Minta et al., 1989). The **Mn^{2+}-fluo-3** complex is eight times as fluorescent as the metal-free dye but only **one-fifth** as fluorescent as the **Ca^{2+}-fluo-3** complex.

Fluo-3/AM was prepared as a 20 mM stock in DMSO. 5 µl of stock solution was added to1 ml external saline containing 1.5 mM Ca^{2+} and 2 mM Mg^{2+} and cells incubated in this solution for 30 to 40 min. Control experiments using ionomycin showed that dye-loading is consistent. After rinsing, the cells were transferred into fresh saline without indicator and left to rest for at least 30 min. to allow esterases to cleave the Fluo-AM ester bond. Dye-loaded cells were imaged on an Olympus Fluoview 300 laser scanning microscope using a 20X water immersion objective (n.a. 0.8). Images were acquired at a rate of 30 frames per min. During analysis, individual cells were delineated as regions-of-interest and the average fluorescence intensity within each region was measured as a function of time. Results are reported as the fractional change in fluorescence intensity ($\Delta F/F$).

External solutions used in imaging experiments (in mM; pH adjusted to 7.2; osmolarity = 290 to 310 mosm):

- Standard (in mM): 140.0 NaCl, 2.8 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 Hepes, 10 glucose (pH 7.4).
- 0 Ca^{2+}/0 Mg^{2+}: 145 NaCl. 2.8 KCl, 10 Hepes, 10 glucose.
- 0 Ca^{2+}/2 Mg^{2+}: 140 NaCl, 2.8 KCl, 2 $MgCl_2$, 10 Hepes, 10 glucose.
- 2 Ca^{2+}/0 Mg^{2+}: 140 NaCl, 2.8 KCl, 2 $CaCl_2$, 10 Hepes, 10 glucose.

Whole-Cell Patch Clamp

Patch clamp experiments were performed in the tight-seal, whole-cell configuration at 23–26°C. Patch pipettes made of borosilicate glass (KIMAX) had resistances between 2–4 Mohm when filled with the standard intracellular solution (see below). Current recordings were acquired by the computer-based patch-clamp amplifier system, EPC-9 (HEKA). Voltages were corrected for a liquid junction potential of 10mV between external and internal solutions (glutamate used as intracellular anion). Currents were filtered at 2.9 kHz and digitized at 100 µs intervals. Capacitive currents and series resistance were determined and corrected using the automatic capacitance compensation of the EPC-9. Immediately following whole-cell break-in, 50-ms voltage ramps from –100 mV to +100 mV were delivered

Mn^{2+} entry via mTRPM7 channels can be resolved at external Mn^{2+} concentrations as low as 1µM

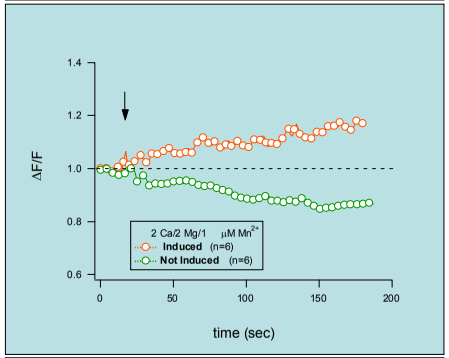

Figure 1. Mn^{2+} influx observed on addition of 1 µM external Mn^{2+}

Fluorescence measurement of Mn^{2+} influx with fluo-3. Each trace represents the average of 6 cells. Cells were loaded with fluo-3/AM and bathed in saline containing 2 mM Ca^{2+} and 2 mM Mg^{2+}. At the arrow, the saline was changed to one containing 1 µM Mn^{2+} in addition to 2 mM Ca^{2+}, 2 mM Mg^{2+}. The fractional change in fluorescence intensity is shown for cells induced to overexpress mTRPM7 and for cells that were not induced. The fluorescence decrease in uninduced cells is similar to what is seen in control experiments and is the result of photobleaching/dye fade.

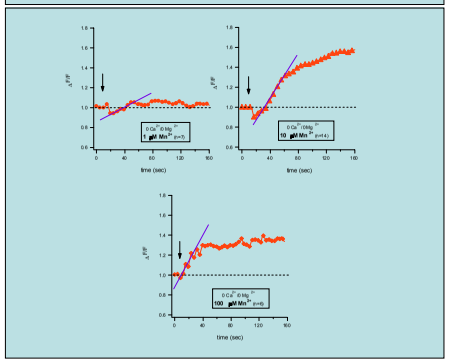

Figure 2. The rate of Mn^{2+} influx increases as Mn^{2+} concentration is increased in the range of 1 to 100 µM

Cells expressing mTRPM7 were loaded with fluo-3/AM and bathed in saline containing no added Ca^{2+} or Mg^{2+} (nominal 0 divalent solution). Mn^{2+} at a concentration of 1, 10 or 100 µM was added at arrow in each panel and the relative increase in fluorescence intensity is plotted as a function of time. Straight lines, drawn by eye, were added to the early part of the fluorescence signal to emphasize the increasing rate of change as the external Mn^{2+} concentration was increased. Traces represent the average of n cells.

Solutions used for whole-cell patch clamp

- **Standard external solution (in mM):** NaCl 140, KCl 2.8, HEPES-NaOH 10, $CaCl_2$ 1.5, $MgCl_2$ 2, CsCl 5, glucose 10; pH is adjusted to 7.2 with NaOH; osmolarity is measured and typically ranges from 295 to 315 mOsm.
- **Application solutions:** $CaCl_2$ or $MgCl_2$ are omitted from the standard solution described above; pH is adjusted with NaOH and osmolarity measured and adjusted with NaCl (such that in some cases final NaCl is 145 mM); in cases where Mn^{2+} is used, appropriate amount of 1M stock $MnCl_2$ is added before pH and osmolarity adjustments.
- **Internal solutions (in mM):** Cs-glutamate 140, NaCl 8, HEPES-CsOH 10, Cs-EGTA 10, $CaCl_2$ 2.5-3.5; pH is adjusted to 7.2 with CsOH, osmolarity is measured and typically ranges from 300 to 320 mOsm. When needed, calculated amounts Mg^{2+} or Mn^{2+} are added from 1M stocks, pH adjusted and osmolarity measured.

Free concentrations of divalents in the internal solution at 25°C (MaxChelator)

	Concentration (mM)	Free [ion]	Free Ca^{2+} (nM)
Ca^{2+}	2.5		56.1
Mg^{2+}	1	737 µM	58.1
Mn^{2+}	0.1	0.1 nM	56.8
	1	1.2 nM	64.7
	2	2.8 nM	76.5

Interactions between Ca^{2+} and Mg^{2+} ions in the pore modulate ion flux via TRPM7 channels

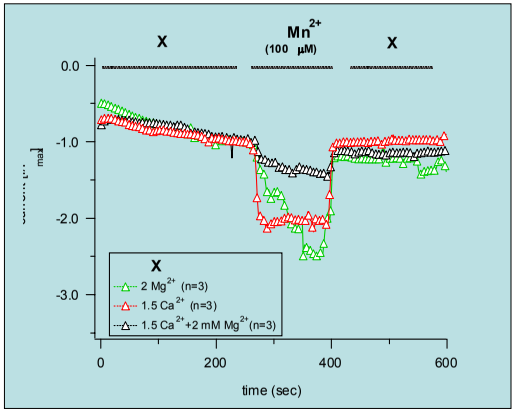

Figure 3. Substitution of external Ca^{2+} and Mg^{2+} by Mn^{2+}

Whole cell currents were recorded in HEK-293 cells induced to overexpress mTRPM7. The cells were bathed in one of three external salines that differ in divalent ion composition: (i) saline containing 2 mM Mg^{2+}/0 Ca^{2+} (green), (ii) saline with 1.5 mM Ca^{2+}/0 Mg^{2+} (red), or (iii) saline with 1.5 mM Ca^{2+}/2 mM Mg^{2+} (black). At the time indicated, the cells were exposed to a low divalent saline containing Mn^{2+} (100 µM) as the only divalent ion for 2 min. After this the saline solution was restored to the original saline.

Inward current increases immediately when saline containing 1.5 mM Ca^{2+} is replaced with saline containing 100 µM Mn^{2+} (red). The increase is constant as long as the Mn^{2+} saline is present and the current returns to the original level immediately when 1.5 mM Ca^{2+} is re-introduced.

Inward current also increases when saline containing 100 µM Mn^{2+} as the only divalent ion replaces Mg^{2+} (green). In this case, the increase develops more gradually and the final current at the end of the 2-min application is larger.

In the third condition, application of Mn^{2+} saline to cells previously exposed to both Ca^{2+} and Mg^{2+} (black), the increase in inward current on introduction of Mn^{2+} has intermediate properties. We draw two conclusions from these results: 1) Mn^{2+} can compete with Ca^{2+} and Mg^{2+} for entry via the channel, 2) Mn^{2+} displaces Ca^{2+} more easily than it displaces Mg^{2+}. Taken together the results suggest competition between Ca^{2+}, Mg^{2+} and Mn^{2+} at sites within the channel.

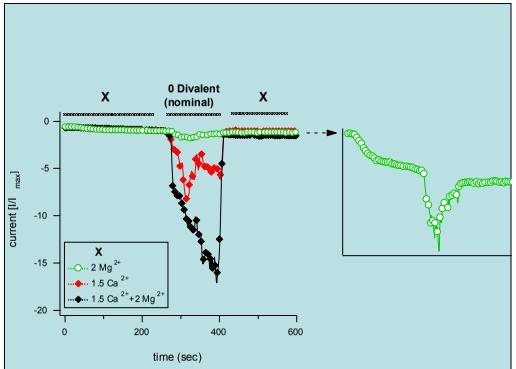

Figure 4. Inward current increases on substitution of Na^{+} for external Ca^{2+} and Mg^{2+}

Saline containing the normal concentration of Na^{+} but without Ca^{2+} or Mg^{2+} (0 Divalent) was applied at the time indicated. Averaged current measured at –80 mV is plotted as function of time (each trace represents the average of 3 cells). This experiment exposes a complicated pattern of ion-ion interactions involving Ca^{2+} and Mg^{2+}. When both Ca^{2+} and Mg^{2+} were initially present in the bath (black trace), substitution with Na^{+} results in a rapid initial increase in inward current and the current continues to increase during the two minute application.

Surprisingly, inward current does not increase to the same extent when the bath initially contained either Mg^{2+} (green) or Ca^{2+} (red) alone. This was especially true when cells were initially bathed in Mg^{2+} saline. When Na^{+} was substituted for Mg^{2+}, the inward current increased by only 2-fold (magnified in the right panel). When only Ca^{2+} was present before the application of 0 Divalent saline, the increase in inward current was larger but not as large as when both Ca^{2+} and Mg^{2+} were present before the switch to 0

Internal Mn^{2+} exerts two disparate effects on TRPM7

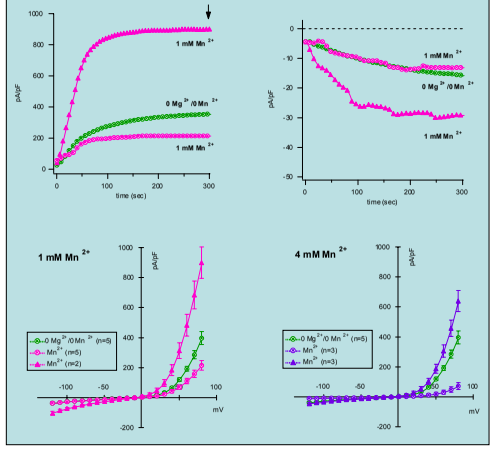

Figure 5. Effects of internal Mn^{2+} on TRPM7

Cells overexpressing mTRPM7 were dialysed with standard internal solution supplemented with different concentrations of Mn^{2+}. Average current densities measured at +80 mV (upper left) and –80 mV (upper right) are plotted over time. Two separate responses are clearly seen. Of seven cells perfused with 1 mM added Mn^{2+}, five showed reduced currents, two had highly augmented currents (pink traces). Sister cells perfused with a solution without added Mn^{2+} nor Mg^{2+} do not show this response (green trace). A step protocol (-120 mV to +80 mV) from a 0 mV holding potential was applied after 300s (marked by arrow in upper left panel). I/V curves averaged from this step protocol are shown in the bottom two panels for the same cells perfused with 1 mM Mn^{2+} (left) and cells perfused with 4 mM Mn^{2+} (right). In the latter condition, half the cells showed current inhibition; the other half had increased currents. The effects of internal Mn^{2+} show some voltage-dependence. Regardless of whether current density increases or decreases, effects of internal Mn^{2+} are more pronounced at depolarized voltages.

SUMMARY

The purpose of this study is to understand the permeation of trace metals via TRPM7 and the regulation of trace metal influx by the abundant metal ions Mg^{2+} and Ca^{2+}. First we sought to demonstrate trace metal influx at concentrations similar to what would be encountered in the organism and in the presence of competing ions. In an earlier study where entry of trace metals through TRPM7 was shown, metal ions were applied at high concentrations (at least 1 mM), far above what would be encountered in a physiological setting (Monteilh-Zoller et al., 2003). With our imaging experiments, we demonstrate that Mn^{2+} entry through these channels can be resolved at external Mn^{2+} concentrations as low as 1µM in the presence of normal, or even slightly elevated, external Ca^{2+} and Mg^{2+}. From this it is apparent that TRPM7 channels can represent a significant source of Mn^{2+} influx under conditions approaching the physiological levels of circulating Mn^{2+} and in the presence of much higher concentrations of competing divalent ions. These experiments make the additional point that Mn^{2+} influx occurs under conditions of membrane voltage and internal and external Mg^{2+} concentration where TRPM7 channels are substantially inhibited (Kozak and Cahalan, 2003).

It seems reasonable to expect that trace metal ions will interact with internal and external metal ion regulatory sites as they transit the TRPM7 channel and that ion-ion interactions at these sites will be important to the regulation of trace metal entry. Using whole-cell patch clamp, we explored Mn^{2+} influx in the presence of either Ca^{2+} or Mg^{2+} and in the presence of both. In agreement with the imaging data, our results indicate that Mn^{2+} can compete with Ca^{2+} and Mg^{2+} for entry through TRPM7 and that Ca^{2+} is more easily displaced than Mg^{2+}. We also looked into substitution of external Ca^{2+} and Mg^{2+} by Na^{+}. Surprisingly, we found that Na^{+} appears to enter TRPM7 more readily when both Ca^{2+} and Mg^{2+} are initially present, than when either is present alone. These results suggest a complex ion-ion interaction between Ca^{2+} and Mg^{2+} whereby one ion destabilizes the other's interaction with its binding site within the channel pore.

Finally, we examined the effects of internal Mn^{2+} on TRPM7. It has been reported previously that internal Mn^{2+} blocks Mg^{2+}-inhibited currents, MIC (Kerschbaum, et al., 2003). In heterologously expressed mTRPM7, we found perfusion with internal Mn^{2+} to increase current density in some cells and reduce it in others. These disparate responses could be due to a variety of reasons including the metabolic state of the cell. Further studies are certainly needed.

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