Depletion of plasma membrane-associated phosphoinositides mimics inhibition of TRPM7 channels by cytosolic Mg²⁺, spermine and pH

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Running title: PIP₂ depletion mimics TRPM7 inhibition by Mg²⁺ and pH

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ABSTRACT

Transient receptor potential cation channel subfamily M member 7 (TRPM7) is an ion channel/protein kinase belonging to the TRP melastatin and eEF2 kinase families. Under physiological conditions, most native TRPM7 channels are inhibited by cytoplasmic Mg²⁺, protons and polyamines. Currents through these channels (I_{TRPM7}) are robustly potentiated when the cell interior is exchanged with low Mg²⁺-containing buffers. I_{TRPM7} is also potentiated by phosphatidylinositol bisphosphate (PI(4,5)P₂) and suppressed by its hydrolysis. Here we characterized internal Mg²⁺- and pH-mediated inhibition of TRPM7 channels in HEK293 cells overexpressing WT voltage-sensing phospholipid phosphatase (VSP) or its catalytically inactive variant VSP-C363S. VSP-mediated depletion of membrane phosphoinositides significantly increased channel sensitivity to Mg²⁺ and pH. Proton concentrations that were too low to inhibit I_{TRPM7} when the VSP-C363S variant was expressed (pH 8.2) became inhibitory in WT-VSP-expressing cells. At pH 6.5, protons inhibited I_{TRPM7} both in WT- and VSP-C363S-expressing cells, but with a faster time course in the WT-VSP-expressing cells. Inhibition by 150 µM Mg²⁺ was also significantly faster in the WT-VSP-expressing cells. Cellular PI(4,5)P₂ depletion increased the sensitivity of TRPM7 channels to the inhibitor 2-APB which acidifies the cytosol. Single substitutions at Ser-1107 of TRPM7, reducing its sensitivity to Mg²⁺, also decreased its inhibition by spermine and acidic pH. Furthermore, these channel variants were markedly less sensitive to VSP-mediated PI(4,5)P₂ depletion than WT. We conclude that the internal Mg²⁺-, polyamine- and pH-mediated inhibition of TRPM7 channels is not direct but, rather, reflects electrostatic screening and resultant disruption of PI(4,5)P₂ channel interactions.

INTRODUCTION

TRPM7 (Channel Kinase 1 (ChaK1)) and the closely related TRPM6 (Channel Kinase 2 (ChaK2)) are unusual membrane proteins containing a TRP channel and a serine/threonine kinase domain (1-4). TRPM7 is highly expressed in cells involved in immunity, such as
lymphocytes, macrophages, microglia and mast cells, various cancer cell lines (5-11) as well as cardiac and vascular smooth muscle and the kidney (12-15). Commonly used cell lines HEK293, COS7, CHO-K1, HeLa and others express substantial outwardly rectifying cation currents mediated by TRPM7 (16-19). A salient feature of TRPM7 channels is their sensitivity to cytoplasmic Mg\(^{2+}\), protons and polyamines (6,7). Mg\(^{2+}\) inhibits Jurkat T-cell TRPM7 channels with a biphasic concentration dependence involving \(~10\ \mu M\) and \(~165\ \mu M\) IC\(_{50}\) inhibitor sites (20). Biphasic Mg\(^{2+}\) dependence was also described for heterologously expressed murine TRPM7 channels (21). By contrast, intracellular protons inhibit with a single pK\(_{a}\) of 6.3 (20). The mechanism of inhibition by these cations has remained a mystery since it was first described. We showed that Mg\(^{2+}\) is not unique in its inhibitory actions and that other divalent (Mn\(^{2+}\), Ca\(^{2+}\), Ba\(^{2+}\)) and trivalent (La\(^{3+}\)) metal cations inhibit in a similar fashion (22). Polyamines spermine (+4 charge), spermidine (+3) and putrescine (+2) inhibit native TRPM7 channels with potencies matching their overall positive electrostatic charge. Neomycin and poly-lysine, large polyvalent cations known to sequester anionic phospholipids (23), are also effective inhibitors (7). An alternative view proposes that inhibition of channel activity by Mg\(^{2+}\) reflects binding of Mg-ATP and other Mg-nucleotides to the C-terminal kinase domain, which somehow communicates this event to the pore and closes it (24-27). In support of this mechanism, channel Mg\(^{2+}\) sensitivity was reduced in cells from TRPM7 kinase-inactivated transgenic mice compared to WT (28).

In whole cell recordings, internal protons and polyvalent cations reduce TRPM7 current amplitude without altering its voltage dependence (6). By contrast, Mg\(^{2+}\) and polyamines applied on the extracellular side produce a rapid voltage-dependent channel pore block (6,29,30). Tonic blockade of inward monovalent current by external Ca\(^{2+}/\)Mg\(^{2+}\) gives rise to the characteristic steep outward rectification of \(I_{\text{TRPM7}}\) (22).

At the single-channel level, inhibition by Mg\(^{2+}\) is a combination of two effects: a gradual reduction of the number of conducting channels and an abrupt, \(~20\%\) drop in the unitary monovalent (39 pS) conductance, seen only with higher Mg\(^{2+}\) concentrations (\(>100\ \mu M\)) (31). In some patches TRPM7 channels were not sensitive to low micromolar Mg\(^{2+}\), whereas in others Mg\(^{2+}\) sensitivity increased upon repeated Mg\(^{2+}\) additions, a phenomenon we termed use-dependence (i.e. sensitization). We hypothesized that in whole-cell recordings, current rundown in presence of Mg\(^{2+}\) reflects a gradual increase in the channels’ sensitivity to Mg\(^{2+}\), akin to the sensitization observed in cell-free patches (31). Current rundown follows the depletion of phosphoinositides in the channel vicinity when ATP is absent (e.g.,6,32)) and can be prevented simply by reducing the Mg\(^{2+}\) concentration to nanomolar, without supplying exogenous phospholipids (7). Presumably, the role of ATP here is to enable replenishment of PIPs by endogenous phospholipid kinases (33-36). Rundown is commonly seen if micromolar or higher concentrations of Mg\(^{2+}\) or spermine are present in the internal solutions (7).

Depletion of membrane PI(4,5)P\(_2\) (below referred to as PIP\(_2\)) by phospholipase C (PLC) \(\beta\) and \(\gamma\) inhibits, while exogenous PIP\(_2\) activates TRPM7 channels (7,32,34). Expression of a heterologous protein that dephosphorylates plasma membrane PIPs at 5' position, the voltage-sensing phosphatase (VSP) (37,38) suppressed TRPM7 channel activity (39).

We previously proposed that inhibition by high internal Mg\(^{2+}\), polyamines and acidic pH represents screening (electrostatic shielding) of negative charges on the phospholipid co-factors of these channels without directly demonstrating this (7). Here we investigated if depletion of PIP\(_2\) by expressing VSP is sufficient to mimic inhibition of TRPM7 channels by these cytosolic cations. We find that PIP\(_2\) depletion significantly increases sensitivity of TRPM7 channels to Mg\(^{2+}\) and protons, in agreement with our hypothesis that these ions act by screening the negative charges of PIP\(_2\) phosphates. Sensitivity to propionate or 2-aminoethyl diphenyl borinate (2-
APB), an inhibitor that acidifies the cytosol (40), is also significantly augmented by PIP$_2$ depletion. TRPM7 Ser1107 (41) mutants, which were reported to be Mg$^{2+}$-insensitive, were also less sensitive to spermine and pH. Importantly, the same mutants (S1107E and S1107R) were significantly less sensitive to PIP$_2$ depletion than WT channels. These observations revealed that inhibition by internal Mg$^{2+}$ and other cations shares a common mechanism and is dependent on cellular PIP$_2$ levels.

RESULTS

Effect of VSP expression on Mg$^{2+}$ sensitivity of native TRPM7 channels

HEK293 cells express significant magnesium-inhibited cation (MIC) currents representing TRPM7 channel activity (20,30). We took advantage of the ease of transfecting this cell type to type the effects of VSP-mediated PIP$_2$ depletion on endogenous TRPM7 channel activity. We compared TRPM7 channel currents in HEK cells transfected with WT (active) and C363S mutant (inactive) CiVSP (38,42). Fig. 1 shows current-voltage (I-V) relations obtained with 10 µM and 150 µM free [Mg$^{2+}$] in cells expressing WT and C363S VSP. I-Vs were unchanged by VSP expression or by Mg$^{2+}$ (Fig. 1A, B, D, E). In GFP+ cells transfected with C363S, there was no noticeable current reduction over time with 10 µM Mg$^{2+}$, (Fig. 1A), whereas in WT VSP expressing cells, the current declined during the course of the experiment (Fig. 1B, C).

150 µM [Mg$^{2+}$], by contrast, was sufficient to inhibit TRPM7 channels in all tested WT and the majority of C363S expressing cells (Fig. 1D-F). Time courses for 10 µM and 150 µM internal Mg$^{2+}$ are shown in Fig. 1C and F: in the absence of PIP$_2$ depletion (i.e. C363S expression) currents developed slowly after whole cell configuration was established and usually reached a maximum 2-6 minutes later (addressed in more detail in Fig. 2). Current suppression by Mg$^{2+}$ could be well fitted with single exponential decay functions (Fig. 1C, F).

We further analyzed the primary data presented in Fig. 1 by dividing the current measured at 6 min by the maximum attained amplitude for each cell. This ratio gives a simple measure of Mg$^{2+}$-induced current decay that follows the initial rise phase (18). It is a single-cell, “steady state” alternative to population measurements of I$_{\text{max}}$ commonly used in studies of TRPM7 channel Mg$^{2+}$ dependence (e.g. (20,24). In Fig. 2A, B, I$_{\text{min}}$/I$_{\text{max}}$ ratios for individual cells at two Mg$^{2+}$ concentrations are presented, grouped according to the VSP variant expressed. These data, summarized in Fig. 2C, demonstrate that when PIP$_2$ was not depleted (C363S VSP transfection), TRPM7 currents did not decay noticeably at 10 µM Mg$^{2+}$ during our recordings. By contrast, expression of WT VSP and consequent PIP$_2$ depletion resulted in a drastic sensitization of the channels to Mg$^{2+}$: ~50.8 to 61.1% of the maximum current was inhibited after 6 min of dialysis. WT VSP expressing cells that showed less decay, most likely represent a subpopulation where the enzyme activity was low, leaving PIP$_2$ levels high (Fig. 2A). Alternatively, these cells may express high levels of endogenous PI-5-kinase enzymes thought to be responsible for PIP$_2$ replenishment (36).

The higher 150 µM Mg$^{2+}$ concentration was sufficient to decrease currents in the majority of C363S-expressing cells (Fig. 2B). The decrease was more pronounced in WT VSP expressing cells: on average 78.9% of the current compared to 34.4% for C363S (Fig. 2B, C). In a minority of C363S-expressing cells 150 µM was ineffective, producing no current reduction, whereas such Mg$^{2+}$-insensitive population was absent amongst WT VSP expressing cells (Fig. 2B). In WT VSP expressing cells percent remaining current was indeed higher at 10 µM (black) than 150 µM Mg$^{2+}$ (red) (Fig. 2D), suggesting that current decay is faster at higher Mg$^{2+}$ concentrations.

WT VSP-expressing cells showed highly variable current amplitudes, even in the same batch of cells (see scatter plots in Fig. 2A). This variability may be due to the heterogeneity of HEK cell resting membrane potentials and
consequent differences in basal lipid phosphatase activity during culturing (see Discussion). Since culturing, transfection, and voltage protocols were identical for all cells, it is unlikely that degree of VSP activation during the recording was the cause of observed disparity (36). An alternative explanation is that not all GFP+ cells express equal amounts of VSP, since in the transfected cell, GFP and VSP are synthesized from one transcript as separate proteins (see Experimental Procedures).

We next compared the decay phases of the current in WT VSP expressing cells for different Mg\(^{2+}\) concentrations (see Fig. 1C, F). The decay time constant (\(\tau\)) was significantly lower with increased internal Mg\(^{2+}\) concentrations (Fig. 2E, F). Thus, at increased Mg\(^{2+}\) concentrations the apparent onset of inhibition was accelerated in PIP\(_2\)-depleted cells.

Exponential decay time constants for WT (\(n = 12\) cells) and C363S expressing cells (\(n = 8\) cells) were compared for 150 \(\mu\)M Mg\(^{2+}\) but were not statistically different (mean ± SE for WT and C363S were 85.13 ± 18.40 and 48.90 ± 5.81, respectively; Student’s t test \(p = 0.315\)).

We next focused our attention on the rising phase of TRPM7 current and its dependence on PIP\(_2\) levels. To this end, times required to reach maximum were examined in WT and C363S expressing cells as a measure of the rate of current development after break-in using two Mg\(^{2+}\) concentrations. Unexpectedly we found that rise times were shorter in PIP\(_2\)-depleted than in control cells. Thus, at 10 \(\mu\)M Mg\(^{2+}\), mean rise time was ~6 min in control (C363S) but only ~2 min in PIP\(_2\) depleted (WT) cells, a 3-fold change (Fig. 2G, L). At 150 \(\mu\)M [Mg\(^{2+}\)], times to reach maximum were decreased in both C363S and WT expressing cells and the difference was not statistically significant (Fig. 2H). This suggested that the effect of increasing [Mg\(^{2+}\)] on the time to reach maximum current can also be mimicked by PIP\(_2\) depletion. The time to reach maximum current in each cell is set by a competition between the rising and the decay phase, i.e. if inhibition is accelerated it will overwhelm the rising phase and manifest as an earlier drop in current magnitude.

In order to determine if the shorter times to maximum in PIP\(_2\) depleted cells reflected a faster current activation, we fitted the rise times with monoexponential functions for C363S and WT VSP expressing cells. We limited our analysis to WT VSP expressing cells that showed decay and C363S expressing cells that showed no decay in current. At 10 \(\mu\)M Mg\(^{2+}\) the time constants were 94.01±19.03 for C363S and 72.9±12.36 for WT VSP (mean ± SEM). Thus, there was a tendency for PIP\(_2\) depletion to accelerate the initial rise of current which was not statistically significant, however (Student’s t test). For 150 \(\mu\)M, such comparison was not performed because the current did not increase in most WT VSP expressing cells (Fig. 1). We conclude that the reduction in time to maximum (Fig. 2G-I) is primarily due to an effect on the decay phase rather than the initial potentiation of the current.

**Effect of PIP\(_2\) depletion on pH regulation and 2-APB sensitivity of TRPM7 channels**

Next, we investigated if dependence of native TRPM7 channel activity on cytosolic pH is also governed by cellular PIP\(_2\) levels, as previously hypothesized (7, 20). We tested two internal solutions which were fixed at acidic (6.5) and basic (8.2) pH values. pH 8.2 (corresponding to [H\(^+\)] ≈ 10 nM) did not suppress TRPM7 channels in control C363S but became inhibitory in WT VSP-transfected cells (Fig. 3) (20). In C363S-expressing cells TRPM7 channel current decayed slowly at pH 6.5 ([H\(^+\)] ~ 1 \(\mu\)M) (Fig. 3A). In WT VSP expressing PIP\(_2\)-depleted cells current reduction at pH 6.5 was markedly faster (Fig. 3A, B). The internal free Mg\(^{2+}\) concentration in these experiments was held constant at 100 nM, a low concentration that prevents channel rundown (31, 40). 2 min or 6 min after reaching the maximum, remaining TRPM7 currents were significantly lower in WT VSP compared to C363S expressing cells (Fig. 3B, C).

A two-way ANOVA analysis performed using TRPM7 current amplitudes remaining 2 min after reaching maximum (Fig. 3B), showed a significant interaction between PIP\(_2\) depletion
and inhibitory effect of protons (p<0.0001). Thus, decreasing cytoplasmic pH had a higher inhibitory effect on TRPM7 channels that were PIP2-depleted (i.e. WT VSP expressing cells) compared to non-depleted channels (C363S VSP).

In order to examine the effect of cytoplasmic acidification on TRPM7 channels in the absence of rundown, we employed the perforated-patch recording configuration which does not perturb the cellular Mg2+. In perforated-patch TRPM7 channels do not run down, presumably because PIP2 is not depleted. We transfected HEK cells with mTRPM7 and studied its dependence on acidification induced by application of sodium propionate, a salt of a weak acid (7). As shown in Fig. 4A, repeated applications of 20 mM propionate resulted in reversible inhibition of TRPM7 currents. The extent of inhibition did not change significantly over time. We then performed the same experiment in whole cell mode with 3 µM Mg2+ in the pipette (Fig. 4B). In this case, repeated application of 20 mM propionate resulted in progressively stronger inhibition of the current. 3 µM Mg2+ is sufficient to support TRPM7 current rundown, likely involving the high affinity Mg2+ inhibitor site (20). In whole cell recordings with lower (400 nM) Mg2+ concentration, which does not support rundown, repeated applications of propionate inhibited the current to a similar extent (Fig. 4C). This behavior was similar to that seen in perforated-patch (Fig. 4A). Therefore, PIP2 depletion in whole cell recordings results in stronger inhibition by cytoplasmic protons which is not seen in perforated-patch recordings when PIP2 is not depleted. This is in agreement with experiments described in Fig. 3, where PIP2 was depleted by VSP expression. Thus, pH and Mg2+ sensitivities of TRPM7 channels are interdependent. The role of low micromolar Mg2+ in channel rundown only becomes apparent when PIP2 is depleted during whole cell dialysis, since the channels do not run down in perforated patch, even though the cytoplasmic Mg2+ is in the millimolar range.

2-APB, a compound widely used in ion channel research, inhibits TRPM7 channels indirectly, by acidifying the cytosol (40). Therefore, we compared inhibition by 100 µM 2-APB in HEK cells to determine if it was dependent on PIP2. In C363S CiVSP-expressing cells this concentration of 2-APB had only a small effect on the current at internal free [Mg2+] of 400 nM (Fig. 5A, C). By contrast, in WT CiVSP-expressing cells 2-APB robustly inhibited the current in 1-2 minutes (Fig. 5B, D). Washout of the drug fully reversed inhibition in C363S but only partially in some WT VSP-expressing cells (Fig. 5B, D). Current decay in WT VSP-expressing cells was substantially slower when 0.05% DMSO was applied without 2-APB (Fig. 5E). On average, 2-APB inhibited 81.8% of current for WT vs. 8% for C363S, quantified at ~3.0 min after application (Fig. 5E). 2-APB inhibition was also increased in cells expressing WT DrVSP, which activates at more depolarized potentials than Ciona enzyme (Fig. 5F). As expected, 2-APB inhibition was voltage-independent (Fig. 5A, B) (40). These results suggest that potency of 2-APB is increased because TRPM7 channels in PIP2-depleted cells are more sensitive to inhibition by protons than in non-depleted controls.

The effect of VSP on TRPM7 channels can occur without depolarization

TRPM7 and TRPM6 channel activity is commonly recorded by applying voltage ramps reaching +80 to +100 mV (see Fig. 1) (6). In order to rule out partial activation of VSP by depolarizations during voltage ramps that briefly reach +85 mV, as has been suggested for TRPM6 (39), we took advantage of the change in TRPM7 I-V relation when external divalent cations are removed: under such conditions TRPM7 becomes semi-linear and allows the measurement of large currents well below 0 mV, where CiVSP should be mostly inactive. We compared TRPM7 current decay using voltage ramps from -100 to +20 mV in WT and C363S VSP expressing cells (Fig. 6A-D). As seen with ramps reaching +85 mV (Fig. 1), native TRPM7 currents declined in minutes in WT but not C363S VSP-expressing cells (Fig. 6E). This observation is in agreement with reduced current magnitudes at break-in (I0)
measured in WT VSP expressing cells (Suppl. Fig. S1A, C) and suggests that VSP is active and can deplete PIPs in HEK cells even without depolarizing voltages.

HEK cells have reported membrane potentials of -40 to -50 mV (43), we therefore sought to evaluate VSP effects under depolarizing, high K+ conditions. Elevating the external [K+] from 5.3 mM of the normal RPMI medium to 25.3 mM is expected to shift the K+ equilibrium potential by +41 mV. Break-in current amplitudes in WT VSP transfected cells grown in low (15 cells) and high [K+] (10 cells) that exhibited current decay were not significantly different (Student’s test, p=0.05) (Fig. 6F). We observed no differences in VSP-induced current decay in cells grown in 5.3 or 25.3 mM [K+], either (data not shown). Mean I_max was higher for C363S than WT VSP expressing cells at both K+ concentrations (p<0.05) (Fig. 6F). These results suggest that either HEK cell membrane is depolarized already or there is significant lipid phosphatase activity that does not require depolarization. To address this question, we measured the resting membrane potentials of HEK cells using perforated-patch, obtaining mean ± SD values of (in mV) -59.7 ± 2.1, -47.5 ± 2.7, -34.8 ± 1.7, -32.4 ± 1.2, -33.9 ± 2.4, -27.8 ± 0.9 (6 cells), similar to published values. Cells with more hyperpolarized membrane potentials exhibited a prominent outward K+ current upon switching to voltage clamp (not shown).

Zebrafish VSP (DrVSP) is activated at more depolarized membrane potentials than its Ciona ortholog (44). The half-activation voltage of DrVSP is 94.27 ± 6.83 mV compared to 62.9 ± 4.5 mV for CiVSP (45). Expression of WT DrVSP in HEK cells resulted in a decay of native TRPM7 currents using -100 - +20 mV ramps, albeit at a slower rate (Fig. 6G). By contrast, currents did not decay in cells transfected with inactive DrVSP C302S mutant (Fig. 6G). Collectively, the data presented in Figs. 6 and Suppl. Fig. S1 (see also Fig. 5) strongly suggest that at least some VSP-induced PIP2 depletion occurs at negative membrane potentials and does not require depolarization.

**Point mutations reducing channel sensitivity to Mg2+ also reduce its sensitivity to spermine and pH**

Ser1107 lies immediately outside of the TRP domain (Fig. 7A) of TRPM7 and was reported to make Mg2+-insensitive ion channels when substituted with glutamate (41). This serine residue is conserved in the human TRPM7 and in the closely related TRPM6 channel. In view of our proposal that polyamines and pH exert inhibition of TRPM7 channels through the same mechanism as Mg2+ (see Introduction), we tested various S1107 mutants for their sensitivity to spermine and pH. We first tested the S1107E mutant, originally described as Mg2+-insensitive (41). Indeed, both spermine and protons were less inhibitory for this mutant (Fig. 7B-E). We further examined the 1107 position by mutagenizing this serine to positively charged lysine (K) and arginine (R) instead of negatively charged glutamate. Both K and R mutants also exhibited diminished Mg2+ sensitivity, accompanied by reduced spermine and pH sensitivity (Fig. 7B-E). As a next step, we mutagenized the same serine to alanine (A), glutamine (Q), threonine (T) and aspartate (D). Surprisingly, no correlation between the charge of this residue and channel sensitivity to Mg2+ was observed. However, we found that the size (surface area) of the substituting residue determined the channel phenotype. For example, changing Ser1107 to glutamic acid or glutamine, having similar size but differing in charge, both resulted in Mg2+-insensitive channels (summarized in Fig. 7E).

WT, S1107A and S1107T TRPM7 magnitudes increased after break-in with 0 Mg2+ internal solution, whereas S1107E, S1107R, S1107K and S1107Q currents were at their maximum magnitude at break-in (I0) and did not rise (Fig. 7B, D). On the other hand, WT, S1107A or S1107T mutants showed only very small current rises in the presence of internal 1.7 mM Mg2+, 3 mM spermine and pH 5.5 (Fig. 7 C, D). This shows that the rising phase of TRPM7 current after break-in is due to the removal of inhibitory cations such as Mg2+ or polyamines and protons. Because S1107E, S1107R, S1107K
and S1107Q (gain-of-function (GOF) mutations) are insensitive to these inhibitory cations, they also do not show a rising phase with 0 internal Mg$^{2+}$. S1107C mutant failed to express substantial currents whereas S1107D behaved as the other GOF mutants (data not shown). These experiments demonstrate that Mg$^{2+}$ is not unique in its inhibition of TRPM7 and shares this property with other cations, such as polyamines and protons. Nevertheless, GOF mutations did not completely eliminate the Mg$^{2+}$ sensitivity of TRPM7: at very high free Mg$^{2+}$ concentrations (tested at 2.9 and 4.9 mM) both S1107E and K mutant channels were partially inhibited (data not shown).

**S1107 gain-of-function mutants are less sensitive to PIP$_2$ depletion by VSP**

As a logical next step we investigated the behavior of mutants with diminished Mg$^{2+}$, spermine and pH sensitivity (GOF mutants) by co-expressing CiVSP. We reasoned that if Mg$^{2+}$, spermine and protons inhibit TRPM7 by screening PIP$_2$ negative charges, then depleting membrane PIP$_2$ would mimic this behavior. In other words, Mg$^{2+}$-insensitive mutants may also be VSP-insensitive. Fig. 8A shows whole cell recordings of WT TRPM7 and two GOF mutants with 0 Mg$^{2+}$. As expected, WT current in VSP-expressing cells increased (due to cytosolic Mg$^{2+}$ washout) then decayed over time, due to further PIP$_2$ depletion. By contrast S1107E and R currents lacked a rising phase and decayed significantly less over the same time period (Fig. 8A, B). This experiment demonstrated that channel sensitivity to Mg$^{2+}$, polyamines and protons strongly correlates with their sensitivity to PIP$_2$ depletion, suggesting that they reflect the same process. To explore the sensitivity of TRPM7’s local structure to changes at position 1107, a truncated model of TRPM7 was constructed and used to probe the consequences of changes at position 1107 (Fig. 8C). Replacement of serine with various residues examined here caused very modest solvent exposure changes, 1-4%, for residues within 8 Å of position 1107. This result was anticipated since the software we used finds only the structure’s closest energy minimum and does not allow for substantial structural changes necessary to locate a new global energy minimum. The degree of instability/stability introduced into the structure by each residue change was estimated by calculating the change in the force field free energy of the 1107 residue and neighboring residues (within 8 Å) relative to the native structure (Ser1107). Results are shown in Fig. 8D (see Fig. S2 for full scale) where the total free energy together with electrostatic and non-bonded components are plotted. The total and non-bonded free energies for Gln (106,023 kJ and 106,174 kJ, respectively), Lys (2,445 kJ and 2,433 kJ) and Arg (11,455 kJ and 11,699 kJ) substitutions are very large and unfavorable, indicating an extremely large driving force for structural change. Other substitutions (Asp and Glu) are associated with smaller free energy changes, but are also unfavorable and of substantial magnitude, indicating a driving force for structural changes in these mutants. Lastly, the electrostatic interactions are relatively small in magnitude. These results suggest that with A, S, C, or T at position 1107 the structure is stable, whereas it is not with D, Q, K or R at the same position.

**DISCUSSION**

We have explored the relationship between well-known regulators of TRPM7 channels, membrane phosphoinositides on one hand and cytoplasmic Mg$^{2+}$, spermine and pH on the other. We employed expression of the voltage-sensitive phospholipid phosphatase (VSP) in order to dephosphorylate the endogenous plasma membrane PIP$_2$ and other phosphoinositide substrates, such as P(3,4,5)P$_3$ (46). Our main findings are: 1) low Mg$^{2+}$ and proton concentrations which were not inhibitory in inactive C363S VSP variant expressing cells (10 µM Mg$^{2+}$ and pH 8.2), significantly inhibited the native TRPM7 channel current when PIP$_2$ was depleted by WT VSP activity, 2) at higher concentrations Mg$^{2+}$ (150 µM ) and protons (pH 6.5) were inhibitory in both WT and C363S VSP-
expressing cells but with a faster time course in the former, 3) native TRPM7 current inhibition by 2-APB was significantly more robust in WT Ciona or zebrafish VSP expressing cells, 4) point mutations reducing TRPM7 channel sensitivity to Mg\(^{2+}\) also reduced its sensitivity to spermine and pH, 5) point mutants insensitive to these cations were also less sensitive to VSP co-expression.

Specifically, several parameters were changed by VSP expression: a) extent of current reduction after the initial rise, measured at 5 or 6 min, b) time constant of current decay, c) time to reach maximum. During whole-cell recording with low Mg\(^{2+}\), TRPM7 current develops slowly, reaching maximum amplitude after several minutes of cell dialysis (Fig. 1A-C). It is thought that this rise time reflects removal of cytosolic Mg\(^{2+}\) (which likely occurs in seconds) and other downstream events. Whole cell recordings (without VSP expression) with no Mg-ATP are accompanied by gradual PIP\(_2\) depletion (e.g. (32,47)). In perforated-patch recording, which prevents Mg\(^{2+}\) and ATP loss (7,48), heterologous TRPM7 channel currents were largely time-invariant (Fig. 4A).

Ionized [Mg\(^{2+}\)] in mammalian cells is in the 0.5-1 mM range (49-51). In our view, the initial current development or potentiation represents Mg\(^{2+}\) (and proton) removal from PIP\(_2\) molecules that are bound to the channels in an intact cell since the average pK\(_a\) of PIP\(_2\) is estimated to be near pH 6.3 (52). We found that time to maximum was shortened in PIP\(_2\) depleted cells (Fig. 2). The difference between C363S and WT VSP transfected cells was reduced at the high 150 \(\mu\)M Mg\(^{2+}\) concentration, reflecting the fact that this concentration is sufficient to inhibit TRPM7 even in cells not depleted of PIP\(_2\). High Mg\(^{2+}\) in effect occludes PIP\(_2\) depletion. We also compared the initial current rise phase in C363S and WT VSP expressing cells and found a small reduction in the time constant for WT VSP which was not statistically significant. We conclude from these measurements that the effect of PIP\(_2\) depletion on the rising phase of the current is primarily due to increasing sensitivity of the channels to Mg\(^{2+}\) and accelerated current decay. We do not rule out a direct effect on the initial rise time, which could be due to a faster off-rate for Mg\(^{2+}\) when fewer PIP\(_2\) molecules are bound to the channel. Conversely, the Mg\(^{2+}\) on-rate, underlying current decline, is enhanced for the same reason, i.e. Mg\(^{2+}\) has to screen fewer PIP\(_2\) molecules in order to close the channels. In other words, the channels become more prone to rundown (31). Alternatively, after binding Mg\(^{2+}\) the PIP\(_2\)-Mg\(^{2+}\) complex is dissociated from the channel, which might help explain why inhibition is slow, requiring several minutes for completion (Figs. 1, 2).

Stimulation of Goq-coupled PLC\(_\beta\) resulted in inhibition of TRPM7 channels through PIP\(_2\) hydrolysis per se, since downstream metabolites DAG and inositol trisphosphate had no effect (34). Application of water-soluble PIP\(_2\) analogs rescued rundown channel activity in that and other reports (7,13,34). In our hands the expression of high levels of CiVSP resulted in significant reductions in both break-in and maximum current amplitudes (Suppl. Fig. S1) supporting the view that PIP\(_2\) is a required cofactor for proper TRPM7 channel function.

We used VSP overexpression to take advantage of testing PIP\(_2\) dependence within the physiological range as opposed to applying exogenous water-soluble PIP\(_2\) analogs at very high concentrations. In some cases exogenously applied PIP\(_2\) activated ion channels which were later found to be PIP\(_2\)-insensitive when endogenous phospholipids were depleted by using genetically engineered tools (see (53,54)).

In WT VSP expressing cells the rates of Mg\(^{2+}\)-dependent inhibition could be described by single exponential decay functions. We compared the decay time constants at the two Mg\(^{2+}\)concentrations and found that increasing the internal Mg\(^{2+}\) concentration to 150 \(\mu\)M resulted in significantly lower time constants (Fig. 2). We interpret this effect as faster overall on-rate of Mg\(^{2+}\) when the number of PIP\(_2\) molecules is reduced by VSP. The rate of inhibition by acidic pH of 6.5 was also substantially higher in PIP\(_2\)-depleted cells (Fig. 3). Thus, depletion of PIP\(_2\) sensitized TRPM7 channels to these inhibitors.

We had to calibrate VSP expression levels to avoid greatly reduced TRPM7 currents.
(see Fig. S1). Surprisingly, even without applying long depolarizing steps, which VSP is believed to require for activation (38), PIP2 levels were apparently lowered during culturing transfected cells for several days. This resulted in great variability of all parameters describing inhibition despite our efforts to keep transfection conditions identical in all cases. Such variability could arise from the differences in resting membrane potentials among HEK cells or differences in VSP expression levels. HEK293 cell resting membrane potentials have been estimated to be in the -50 to -30 mV range (55-58). In order to investigate if depolarizing shifts in the resting membrane potential will increase VSP activity, transfected HEK cells were cultured in 25.3 mM KCl containing RPMI medium for 24 hours before recordings were made (Fig. 6F). For HEK cells expressing WT and C363S the break-in and maximum current amplitudes in high and normal K+ (Fig. 6F) were not statistically different, however (Student’s t test). Our own measurements of HEK cell membrane potential in 4.5 mM [K+], showed that it varied between -59 and -27 mV (see Results). This points to the existence of substantial VSP activity at these negative membrane potentials. Accordingly, it has been previously demonstrated that CiVSP enzyme is active over a wide range of membrane potentials achievable in patch clamp experiments (42, 44).

We also looked for acute effects of VSP on TRPM7 channel currents by applying a long (2 s) depolarizing step either to +100 mV or to -10 mV and comparing the speed of current decay but saw no noticeable acceleration of current inhibition with +100 mV steps, a sufficient depolarization for VSP activation, used by other investigators for this purpose (e.g. (35,53)) (data not shown). It appears that in the case of low affinity interactions of PIP2 with channels, the basal activity of VSP is sufficient to account for the observed effects on Mg2+ and pH sensitivity. The command voltage ramp protocol applied in our experiments to measure TRPM7 currents briefly reaches +85 mV (see Experimental Procedures) and may by itself be sufficient to activate VSP. This was proposed for a similar voltage protocol (-120 to +100 mV applied at 1 Hz) used to record TRPM6 (39). Arguing against this scenario is our observation that high efficiency VSP transfection reduced break-in TRPM7 currents for WT compared to C363S VSP expressing cells (Suppl. Fig. S1).

In addition to PIP2, VSP can hydrolyze PI(3,4,5)P3 while generating PI(4)P in the process (46,54,59). While participation of these phospholipids in TRPM7 channel regulation is not ruled out, most experimental evidence points to PIP2 as the primary anionic phospholipid required for TRPM7 activation (39).

Electrostatic interactions with PIP2 have been linked to increased Mg2+ sensitivity of several ion channels. Hydrolysis of PIP2 resulted in a greater degree and speed of TRP5V5 channel inhibition by internal Mg2+ (60). Du and colleagues (61) showed that Mg2+ and pH sensitivity of Kir channels depends on the availability of PIP2 and its interaction with the channels. Accordingly, point mutations strengthening Kir2.3 channel-PIP2 interactions reduced channel inhibition by Mg2+ and protons (compare to our Figs. 7 and 8), whereas those weakening Kir2.1-PIP2 interactions increased the Mg2+ and proton sensitivity. The authors proposed that Mg2+ effect was mediated by stimulation of lipid phosphatases. A dominant negative point mutation in Kir2.1 linked to Andersen syndrome decreased channels’ PIP2 sensitivity, resulting in increased inhibition by Mg2+ (62). PIP2-sensitive KCNE-KCNQ channels are inhibited by intracellular Mg2+ and polyamines and elevated PIP2 concentrations decreased inhibition by these cations (63). It can be summarized that low affinity channel-PIP2 interactions are disrupted by physiological levels of Mg2+, protons and polyamines, whereas high affinity interactions are less sensitive to these cations (36,54,61). In agreement with this view, polyvalent cations with low charge-screening capacity, such as hexamethonium, do not inhibit TRPM7 (7).

We showed previously that inhibition of native TRPM7 channels by 2-APB is not direct but by cytoplasmic acidification induced by this drug (40). In PIP2-depleted cells, same
concentration of 2-APB was significantly more potent than in controls (Fig. 5), in agreement with increased pH sensitivity of TRPM7 channels under these conditions (Fig. 3). Inhibition by 2-APB was considerably faster for WT than for C363S consistent with a faster proton-induced I_{TRPM7} decline (Fig. 3). It would be instructive to test if other TRPM7 modulators that depend on internal Mg^{2+} (64) also demonstrate increased potencies in PIP_{2}-depleted cells.

Several basic residues have been implicated in PIP_{2} sensitivity of TRPM7 channels. Mutagenesis of basic Lys1112, Arg1115 and Lys1125 (triple mutant) located in the TRP domain (see Fig. 7A) to neutral glutamines resulted in non-functional channels (39). Since TRPM7 forms a homotetramer (4,65) these results suggest that mutagenizing these three basic residues in all four subunits completely abolishes channel function. K1112Q TRPM7 (39), however, had low basal activity which could be potentiated by external NH_{4}^{+} to alkalinize the cytosol and neutralize the inhibitory protons (7,39). It is not known if additional PIP_{2} interacting sites exist in TRPM7 protein and if every subunit needs to bind PIP_{2} for the channel to open. For another tetrameric channel binding to three subunits was sufficient to support channel activity (66). The effect of PIP_{2} depletion on TRPM7 channels may either represent multiple PIP_{2} binding sites on each subunit and/or removal of PIP_{2} from an increasing number of subunits comprising the channel. Further experimentation will be needed to answer this question directly.

Ser1107 was described as a key residue mediating Mg^{2+} inhibition of TRPM7 (41) (Fig. 8C): substitution with negatively charged glutamate resulted in channels with greatly reduced Mg^{2+} sensitivity. We investigated what other amino acid substitutions in this position would reduce Mg^{2+} sensitivity (gain-of-function mutations). Surprisingly, substituting S1107 with positively charged arginine (R) or lysine (K) resulted in an identical phenotype (Figs. 7, 8D). Uncharged glutamine (Q) also gave rise to channels insensitive to Mg^{2+} (Figs. 7, 8D). Substitution with alanine or threonine, on the other hand, resulted in channels that behaved like WT. We then tested if these point mutants were sensitive to other positively charged inhibitors such as spermine and protons, finding that the GOF mutants were strikingly less sensitive to both, whereas S1107A and T mutants were inhibited by all three, like the WT channel (Fig. 7).

We further tested S1107 mutants for their sensitivity to PIP_{2} depletion by co-expression with VSP. We found that S1107E and S1107R mutants were also significantly less sensitive to VSP-induced PIP_{2} depletion compared to WT (Fig. 8A, B). In combination, these results suggest that Ser1107 is critical for the sensitivity of TRPM7 to cations, polyamines and PIP_{2} interaction and certain substitutions at this site can disrupt this interaction. Mutations by which PIP_{2}-channel interactions are potentially strengthened (S1107E, R, K, Q) are also more difficult to disrupt by the screening cations. Indeed, we were able to inhibit GOF mutant currents only by raising [Mg^{2+}] to 2.9 mM and higher (see Results). It is notable that the positive regulator of TRPM7 channel activity (PIP_{2}) and the negative regulators (Mg^{2+}, spermine, protons) share the same site of action, suggesting that Mg^{2+}, polyamines and pH exert their inhibitory actions through the same mechanism: electrostatic screening (shielding) of PIP_{2} negative charge (7). We, therefore, predict that neomycin and polylysine, polycations which efficiently interact with PIP_{2}, will also be less potent in GOF mutants compared to WT. We also predict that S1107D, K and Q mutants will behave like E and R, whereas S1107A and T are likely to behave like WT channel in terms of VSP sensitivity. The phenotype of S1107 substitutions does not depend on the charge in that position, since arginine, lysine and glutamate behave similarly. Rather, it depends on the bulk as reflected in the surface area of the substituting amino acid residue (67)-(68). Thus, alanine (surface area of 115 Å^{2}) and threonine (140 Å^{2}) mutants are similar to WT serine (115 Å^{2}), whereas larger side chains (D, Q, E, K, R) with respective surface areas of 150, 180, 190, 200, 225 Å^{2} make the channel insensitive to Mg^{2+} and
other cations (Fig. 8D). Acidic glutamate (190 Å²) and uncharged glutamine (180 Å²) behave similarly, having similar size. The cutoff appears somewhat between 140 and 150 Å² (residues in red in Fig. 8D), since threonine mutant behaved as WT, whereas aspartate was a GOF mutant. Surprisingly, the exposure of residue side chains within the truncated TRPM7 model used here changes very little (1% to 4%) despite variations in the size of the mutant side chains at 1107 (Suppl. Fig. S2). For some mutations at position 1107 (Q, K, R) energies are extremely large and unfavorable. Others (D and E) are more modest but they are also unfavorable and of significant magnitude. This may provide the energetic driving force for a conformational change in the region of residue 1107 which modifies or abolishes PIP₂ interactions with the protein. Contributions from electrostatic interactions are not as great as those from non-bonded (steric) interactions and are even stabilizing in the case of Q and R. This suggests the bulk of side chains rather than their charge is of greatest consequence. In total, modeling supports functional analyses which indicate that A and C mutants retain native activity while D, Q, E, K, R do not (Figs. 7, 8). These findings are unexpected since many publications until now have argued that PIP₂ interacts with basic amino acids of the channel protein (e.g. (39,69-71)). Apparently, the side chain bulk plays an important role in these interactions in the case of TRPM7. We are not aware of other studies implicating amino acid size as a determinant of PIP₂ sensitivity of channels. Whether S1107 directly binds PIP₂ was not determined. It might, for example, function downstream of PIP₂ binding by communicating with the channel gating machinery. It is unlikely to bind Mg²⁺ or other inhibitory cations, however.

S1107 in TRPM7 or its corresponding S1080 in human TRPM6 have not been identified as phosphorylation sites in intact cells so far, suggesting that it influences TRPM7 channel activity by a mechanism independent of TRPM7/6 kinase activity (72,73).

In this paper we have demonstrated that PIP₂ depletion can mimic the inhibitory effects of internal Mg²⁺ and pH on TRPM7 channel activity. Our interpretation of VSP data assumes that PI(4,5)P₂ phospholipid is its main substrate. Several interesting questions about TRPM7 regulation can be addressed in our future experiments: what is the effect of PIP₂ depletion at the single-channel level? Mg²⁺ inhibition consists of a gradual disappearance of conducting channels and a modest, ~20% reduction in unitary conductance (31); will VSP-mediated PIP₂ depletion mimic both of these effects? The latter effect is particularly interesting since it must involve the ion conduction pathway itself. It is also not known if pH and polyamine effects on single-channel TRPM7 activity are similar to Mg²⁺ effects and if the high affinity Mg²⁺ inhibitory site is also PIP₂-dependent. The question of the minimum subunit number required to bind PIP₂ to activate the channel remains open and will allow a more quantitative description of channel inhibition by polyvalent cations.

EXPERIMENTAL PROCEDURES

Cell maintenance and transfection

HEK293 cell line was maintained in RPMI-1640 medium (Lonza, Walkersville, MD) supplemented with 10% heat-inactivated fetal bovine serum (Fisher Scientific, Fair Lawn, NJ) and penicillin/streptomycin (HyClone) in a cell culture incubator (Forma Scientific, Marietta, OH) at 37°C and 5% CO₂ as previously described (20). Cells were grown in 10 cm polystyrene cell culture dishes (USA Scientific, Ocala, FL) and passaged twice a week. Cells plated in 6-well polystyrene plates (USA Scientific) the day before were transfected either with CiVSP or DrVSP cDNA in pIRES2-EGFP bicistronic plasmid vectors provided by Yasushi Okamura, Osaka University, Japan. Inactive C363S (Ciona) and C302S (zebrafish) variants were transfected as negative controls. Transient transfection of plasmid DNA was performed with TransIT-LT1 reagent (Mirus Bio, Madison, WI) according to the manufacturer’s recommendations. 2-3 days after transfection the cells were lifted and transferred to glass-bottom recording chambers. In one series of experiments the transfection mix
was kept in the wells until the start of recordings (~48 hrs) (Suppl. Fig. S1), whereas in the other series it was washed away by changing the culture medium after ~24 hrs. We found that after ~48 hrs of uninterrupted transfection, the break-in (basal) and maximum current amplitudes were both markedly reduced in WT compared to CiVSP C363S transfected cells, resulting in many cells having no detectable endogenous TRPM7 currents (Fig. S1). We, therefore, conducted our experiments using moderate transfection conditions by removing the transfection reagent after ~24 hrs.

Murine TRPM7 coding sequence (18) and its S1107 point mutants in GFP-tagged (in pEGFP-C1 plasmid vector) or untagged version (in pcDNA3) were transfected at 2.5 µg/well in 6 well-plates and recordings were made 2-3 days after transfection. For co-transfection, 2.5 µg of TRPM7-pcDNA3 (WT or S1107 point mutants) and 0.6-0.8 µg of WT Ci-VSP plasmid were used per well, and the majority of recordings were made 2 days after transfection. Occasionally, recordings were made 3 days after transfecting with VSP with similar results.

Site-directed mutagenesis
Serine 1107 (41) of murine TRPM7 in pEGFP-C1 and pcDNA3 vectors was mutagenized to alanine, glutamate, glutamine, cysteine, threonine, lysine or arginine using QuikChange II XL kit (Promega, Madison, WI). All mutations were verified by DNA sequencing (Retrogen, San Diego, CA).

Patch-clamp electrophysiology
Whole cell patch clamp recordings were performed as previously described (6,20). Pipettes were manufactured from patch capillary glass (Warner Instruments, Hamden, CT) using a DMZ Universal (Zeitz Instruments, Martinsried, Germany) or P-1000 micropipette puller (Sutter Instrument, Novato, CA) and had resistances of ~2-4 MΩ. Currents were recorded with an EPC10 patch clamp amplifier and Patchmaster software (HEKA Elektronik, Lambrecht, Germany). On the day of experiment, cells were lifted off polystyrene culture plate and transferred to the recording chamber mounted on the mechanical stage of an inverted microscope equipped with epifluorescence (Nikon, Japan). Successfully transfected cells were identified by their EGFP fluorescence and selected for patch-clamp recording. In several experiments we also recorded from non-fluorescent cells for comparison to rule out nonspecific transfection effects (see Fig. 6F). For recording endogenous TRPM7 currents, the internal (pipette) solution contained (in mM): 106 L-glutamic acid, 8 NaCl, 5 CsF, 10 HEDTA, 10 HEPES acid, pH adjusted to 7.3 with CsOH. For experiments described in Fig. 3, pH of this solution was brought to 6.5 or 8.2 on the day of the experiment. For preparing solutions containing 10 µM and 150 µM free Mg²⁺, glutamic acid and HEDTA concentrations were lowered to 101 mM and 8 mM, respectively. For recordings of overexpressed mTRPM7 and its point mutants (Figs. 7, 8) the internal solution contained (in mM) 112 glutamic acid, 8 NaCl, 5 CsF, 12 EGTA, 10 HEPES acid, 0.09 CaCl₂, pH 7.3 with CsOH. In all experiments (except Fig. 6) the external (bath) solution contained (in mM): 2 CaCl₂, 4.5 KCl, 140 Na aspartate, 10 HEPES-Na+, 3 CsCl, 0.5 glucose, pH 7.3. 1.0 M MgCl₂ and CaCl₂ standard solutions (Sigma-Aldrich, St. Louis, MO) were used for buffer preparation. 20 mM Na aspartate was replaced with 20 mM Na propionate in the standard 2 mM Ca²⁺ buffer to perform the experiments described in Fig. 4.

For Fig. 6, divalent cation free (DVF) external solution was used consisting of (in mM) 140 aspartic acid, 6 HEDTA, 10 HEPES, pH 7.3 (with CsOH) or 140 mM sodium aspartate, 6 HEDTA, 10 HEPES, pH 7.3 (with NaOH). Deionized water (Nanopure, Barnstead, UK) was used in preparation of recording solutions. Osmolalities were measured with a freezing point depression osmometer (Precision Systems, Natick, MA) and adjusted by adding D-mannitol. Free Mg²⁺ concentrations were calculated with Webmaxc software (http://maxchelator.stanford.edu/webmaxc/webmaxc5.htm).

Endogenous HEK cell and heterologous TRPM7 channel currents (20) were evoked by applying 211 ms duration command voltage...
ramps ranging from -100 to +85 mV every 2.5 seconds. In the experiments shown in Figs. 4, 5, 7 and 8, -85 to +85 mV voltage ramps of the same duration and frequency were used, except for a few recordings where 2 s gaps were used (see Fig. 8 legend). Current traces were filtered at 2.9 kHz and digitized at 5 kHz. Data acquired with Patchmaster were saved on the hard drive of a PC for subsequent analysis. For recording monovalent TRPM7 currents in DVF solutions, voltage ramps from -100 to +20 mV were used (Fig. 6). In some cells, currents continued rising slowly after 6 min at ~1.5% per min: these small increases were ignored.

Occasionally, endogenous $I_{\text{TRPM7}}$ amplitudes recovered again after initial inhibition by 150 µM Mg$^{2+}$ (not shown). This rebound phenomenon was only observed in C363S but not WT VSP transfected cells. Such cells were excluded from our analysis.

Membrane potentials of untransfected HEK293 cells were measured using the perforated-patch technique, essentially as previously described (7). Briefly, an aliquot of amphotericin B (Sigma) prepared in DMSO was dissolved in the internal solution containing 55 mM KCl, 50 mM K$_2$SO$_4$, 7 mM MgCl$_2$, 1 mM CaCl$_2$, 10 HEPES, pH 7.3, to a final concentration of ~240 µg/ml. External solution contained 77 mM Na aspartate, 62.5 mM NaCl, 2 mM CaCl$_2$, 4.5 mM KCl, 10 mM HEPES-sodium, 0.5 mM glucose, pH 7.3. Membrane potentials were recorded in current clamp mode continuously for approximately 1-6 minutes (10 kHz sampling rate) with $I_{\text{membrane}} = 0$, after access resistances dropped to 20 MΩ or lower. For recording TRPM7 currents (Fig. 5A), the perforated-patch solution contained Cs$^+$ instead of K$^+$, 7 mM Mg$^{2+}$ present in this solution was sufficient to inhibit TRPM7 currents if whole cell break-in occurred spontaneously.

All experiments were performed at room temperature (~25 °C).

**Molecular structure presentation**

A partial structural model for mouse TRPM7 was developed using homology modeling methods implemented by Phyre2 (74,75). The alpha kinase domain, as well as regions of TRPM7 primary structure for which satisfactory structural homologs could not be identified, are not included in our model. To examine the structural consequences of mutations in the truncated TRPM7 model, it was energy minimized using the GROMOS force field and the amino acid residue at position 1107 in the sequence was modified to alternate residues using Swiss PDB Viewer utilities. The resultant structures were again energy minimized. Threading energies at each amino acid residue of these resulting structures were calculated. For each mutant protein threading energies for residues within 8 Å of position 1107 were used to estimate the energetic consequences of the respective mutation. The solvent-accessible surfaces of each residue in the native and mutated TRPM7 structural models were also determined using SPDBV. Energy calculations were performed using the GROMOS96 43B1 parameter set, without reaction field, as implemented in SPDBV. The resultant structures were modified to alternate residues using Swiss PDB Viewer (SPDBV) (76). In these calculations solvent was not explicitly included.

**Chemicals and data presentation**

L-glutamic acid, HEPES, EGTA and HEDTA were from Acros Organics (Geel, Belgium). D-mannitol, NaOH and DMSO were from Fisher Scientific. All other salts and 2-APB were purchased from Sigma-Aldrich. 200 mM stock solution of 2-APB was prepared in DMSO and diluted in the external recording solution on the day of experiment. Analysis was done by comparing data collected from groups of cells transfected with WT, inactive VSP mutants and/or mTRPM7 constructs and presented as scatter plots and bar graphs of means ± SEM. Patch clamp data curve fitting and graphing was performed using Origin v. 8, 8.6 and 2016 (OriginLab, Northampton, MA). Numbers of cells in graphs are given in parentheses. Statistical differences were compared using ANOVA, Student’s t test, Tukey’s multiple comparisons test.
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CONFLICT OF INTEREST
The authors declare that they have no conflicts of interest with the contents of this article.

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PIP2 depletion mimics TRPM7 inhibition by Mg²⁺ and pH

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**FOOTNOTES**

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**FIGURE LEGENDS**

**Figure 1.** Native TRPM7 channel currents in WT and C363S VSP expressing HEK cells. TRPM7 current-voltage relations with 10 µM (A, B) and 150 µM (D, E) free internal Mg\(^{2+}\) in WT (B, E) and C363S (A, D) CiVSP expressing cells. C, F. Time courses of current development and reduction with internal 10 µM and 150 µM Mg\(^{2+}\). In this and subsequent figures (except figures 8, 9) filled and open symbols represent current amplitudes measured in cells expressing WT and C363S VSP, respectively. Graphs in A, B, C and D, E, F were obtained from the same cells. The declining current amplitude was fitted with a single exponential decay function (C, F). The membrane potential between ramps was held at -60 mV.

**Figure 2.** Mg\(^{2+}\) dependence of native TRPM7 current amplitude and decay rate in VSP expressing cells. A, B. Amplitudes measured in whole cell at 6 min were divided by maximum attained current, set as 100%, in each cell and presented as percentages. Each point represents a ratio of measurements obtained from a single cell. Internal Mg\(^{2+}\) concentrations are shown in the graph. For cells in which the current continued to increase at and after 6 min, amplitudes at t = 6 min were taken as the maximum. C. Summary of experiments in A and B. For the indicated Mg\(^{2+}\) concentrations, mean I\(_{6\text{ min}}\)/I\(_{\text{max}}\) ratio was significantly lower for WT than for C363S VSP transfected HEK cells. Empty and filled bars here and in other figures represent data from C363S and WT VSP expressing cells, respectively. *p<0.002, Tukey’s multiple comparisons test. Two C363S VSP expressing cells in which decay was linear were excluded from analysis. D. Data shown in C represented as percentage of TRPM7 current remaining at 6
min in WT relative to C363S VSP (set as 100%) expressing cells, with free internal Mg\(^{2+}\) of 10 µM (black) or 150 µM (red). E. Scatter graph of exponential decay time constant (tau) values in WT VSP-transfected cells at 10 and 150 µM Mg\(^{2+}\) concentrations. F. Summary of data shown in E. *p<0.05. G, H. Times of whole cell dialysis required to reach maximum current amplitude at the indicated Mg\(^{2+}\) concentrations. Each point represents a measurement obtained from a single cell. I. Summary of experiments in G and H. Mean rise times were shorter for WT than for C363S VSP-transfected HEK cells for 10 µM and 150 µM [Mg\(^{2+}\)]. Time = 0 represents cells that did not show current increase after break-in. * p<0.05. Horizontal lines in figures C, F and I represent arithmetic means.

**Figure 3.** pH dependence of native TRPM7 channels in WT and C363S VSP expressing cells. A. Time courses of TRPM7 current reduction obtained when pH of the internal solution was 8.2 (blue) and 6.5 (red) in cells expressing WT (filled) and C363S VSP (hollow). Currents measured at +84.84 mV were normalized to I\(_{\text{max}}\) for each cell. Only the decay phase of the current is plotted against time. Current inhibition at 2 min (B) and 6 min (C) for pH 8.2 and 6.5 obtained from A. * p<0.001. Exponential decay time constant (tau) for WT VSP at 6.5 was 76.17 ± 1.49 s, R\(^2\)=0.99. Internal [Mg\(^{2+}\)] was ~80 nM in all recordings. Membrane potential was held at -60 mV between ramps.

**Figure 4.** Inhibition of recombinant TRPM7 channel currents by propionate-induced acidification in perforated-patch and whole cell configurations. Perforated-patch (A) and whole cell (B, C) recordings of current inhibition by repeated application of 20 mM Na-propionate in HEK cells expressing WT mTRPM7-GFP. Time courses (left) and I-V relations (right) are depicted. Vertical arrows indicate the time points where I-Vs were obtained. Internal free [Mg\(^{2+}\)] was 3 µM in B and 400 nM in C. Representative recordings chosen from n = 5 (A), 5 (B) and 7 (C) experiments. The membrane potential between ramps was held at 0 mV.

**Figure 5.** Response of native I\(_{\text{TRPM7}}\) to 2-APB. A, B. I-V relations in C363S and WT CiVSP expressing HEK cells immediately before (black) and in the presence of 100 µM 2-APB (red). W/O trace in B refers to washout. C, D. Corresponding time courses of I\(_{\text{TRPM7}}\) amplitude from same cells as in A and B and 0.05% DMSO control. E. Extent of current inhibition by 2-APB and 0.05% DMSO alone in C363S and WT CiVSP expressing cells. F. Extent of current reduction in 100 µM 2-APB, 0.05% DMSO and vehicle control in WT DrVSP expressing cells. Fraction of unblocked current was obtained by dividing I\(_{\text{TRPM7}}\) amplitudes immediately before adding 2-APB or DMSO and after 72 (E) and 60 (F) voltage ramps in their presence. Y = 1 corresponds to no inhibition. *p<0.05, Student’s two-sample t test. Internal free [Mg\(^{2+}\)] was 400 nM and the holding potential was 0 mV.

**Figure 6.** Voltage dependence of VSP effect on native TRPM7 channels. Time courses of inward TRPM7 current (A, B) and I-V (C, D) in divalent cation-free (DVF) Cs\(^{+}\)-based bath solutions. Voltage ramps from -100 to +20 mV were applied. A-D were obtained from HEK cells expressing WT or C363S CiVSP. E. Current remaining after 6 min of whole cell recording, divided by the maximum current attained in the respective cell. Inward current at -100 mV was plotted. Internal free [Mg\(^{2+}\)] was 10 µM. *p<0.005. F. Cells transfected with WT or C363S CiVSP were grown in RPMI-1640 media, containing 5.3 mM KCl or 25.3 mM KCl. TRPM7 current amplitudes at break-in and maximum currents attained. Current measurements taken from non-GFP cells among the C363S VSP transfected cells were used as an untransfected control. Pairs grown in high and low K\(^{+}\) were not significantly different for WT and C363S VSP (t-test, p>0.05). [Mg\(^{2+}\)] = 400 nM. The holding potential was -60 mV. G. TRPM7 current decay in C302S and WT DrVSP transfected cells. Current remaining after 10 min of recording, divided by the maximum current. -100 to +20 mV ramps were used as in A. The internal free [Mg\(^{2+}\)] = 10 µM, external DVF solutions was Na\(^{+}\)-based. Holding potential was 0 mV.
Figure 7.  Mg\textsuperscript{2+}, spermine and pH regulate TRPM7 channel activity through the same mechanism. A. Domain structure of TRPM7 with the position of Ser1107 indicated by arrow. B. Representative time courses of WT and S1107A, S1107E, S1107R, S1107K, S1107Q mutant TRPM7 currents with internal 0 Mg\textsuperscript{2+} (B), 3 mM total MgCl\textsubscript{2} corresponding to calculated 1.7 mM free Mg\textsuperscript{2+}, 3 mM spermine and pH 5.5 (C). In B and C TRPM7 current was normalized to maximum current (I\textsubscript{max}) in each cell. D. Mean maximum TRPM7 current amplitudes divided by break-in (I\textsubscript{0}) current in HEK cells expressing WT, S1107A, S1107E, S1107R, S1107K, S1107Q or S1107T mTRPM7. E. Bar graph showing mean current amplitudes at 5 min normalized to maximum current in each cell. Graphs in D and E were obtained from the same cells. The internal solution contained Mg\textsuperscript{2+}, spermine or acidic pH of 5.5 as indicated. *p<0.05, Student’s two-sample t test. * in E indicates significant differences in I\textsubscript{5min}/I\textsubscript{max} compared to WT at respective Mg\textsuperscript{2+}, spermine or proton concentrations. Experiments were performed with n = 4-11 cells for each condition. For 5 cells with 1.7 mM free internal Mg\textsuperscript{2+} and 3 cells with 3 mM spermine expressing S1107A, the command voltage ramps were applied every 2 instead of 2.5 seconds. The holding potential was 0 mV between ramps.

Figure 8. Ser1107 is involved in mediating TRPM7 sensitivity to PIP\textsubscript{2} depletion by VSP. A. Representative time courses of WT, S1107E and S1107R TRPM7 currents in cells co-transfected with WT CiVSP. TRPM7 current at each time point was normalized to maximum current in the respective cell. Note that unlike WT, for E and R mutants the current does not increase after break-in. B. Bar graph showing current amplitudes 100 ramps (about 4.2 min) after reaching maximum, divided by maximum current amplitude in HEK cells co-expressing WT VSP with WT, S1107E or S1107R TRPM7. Internal solution contained 12 mM EGTA, 0 MgCl\textsubscript{2}. *p<0.0001, Student’s two-sample t test. * indicates significant difference compared to WT. C. Partial structural model of a TRPM7 monomer predicted as described in Experimental Procedures. Transmembrane helices 1 through 6 are colored brown, green, cyan, red, magenta, dark purple, respectively. Pore helix is shown in blue. The arrow indicates residue S1107’s position in the structural model. The cytoplasmic portion of the protein is shown in grey. D. Summary of experimental findings from S1107 mutagenesis. Numbers represent predicted surface areas (Å\textsuperscript{2}) (top) and volumes (Å\textsuperscript{3}) (bottom) of amino acid residues obtained from (67,68). C* denotes S1107C mutant which did not give rise to functional channels. E. Differences in threading energies between native and mutant TRPM7 models at locations within 8 Å of position 1107. Electrostatic (red bars), non-bonding (steric, green) and total (blue) energies were calculated as described in Experimental Procedures.
**Figure 1**

A  C363S (inactive) VSP

B  WT VSP

C  WT VSP

D  C363S (inactive) VSP

E  WT VSP

F  WT VSP
Figure 3

Panel A: Graph showing the change in I/I_max over time (min) for WT and C363S at pH 6.5 and 8.2.

Panel B: Bar graph comparing VSP pH 6.5 and pH 8.2 for C363S and WT.

Panel C: Bar graph comparing VSP pH 6.5 and pH 8.2 for C363S and WT.

* indicates statistical significance.
Figure 4

A  perforated patch

B  whole cell 3 μM Mg$^{2+}$

C  whole cell 400 nM Mg$^{2+}$
Figure 5

(A) Voltage-current (I-V) relationship for C363S and WT A-currents in the absence of 2-APB and with 100 μM 2-APB. The control curve is shown in red. (B) Time course of current activation for C363S and WT A-currents in the absence of 2-APB and with 100 μM 2-APB. The control curve is shown in red. (C) Fraction of unblocked current for CiVSP and DrVSP A-currents in the presence of 2-APB and DMSO. The control curve is shown in red. (D) Fraction of unblocked current for WT A-currents in the presence of 2-APB and DMSO. The control curve is shown in red.

* indicates a significant difference from the control.
Figure 6

A  B  G
C  D

C363S

WT

0 mV
+20 mV

I (pA)
time (s)

I
I

I
max
max
max

V (mV)

C363S

WT

(7)

(6)

C302S

WT

(11)

n.s.

n.s.

n.s.

n.s.

n.s.

n.s.

C363S

WT

0 mV
+20 mV

I
I

max
max
max

V (mV)

C363S

WT

DrVSP

DrVSP
Figure 7

A

B

C

D

E

1.7 mM Mg$^{2+}$

3 mM SPM

pH 5.5

WT TRPM7
S1107A
S1107E
S1107R
S1107K
S1107Q
S1107T

I$_{\text{max}}$/I$_0$

0 Mg$^{2+}$
1.7 mM Mg$^{2+}$
3 mM SPM
pH 5.5

I$_{5\text{min}}$/I$_{\text{max}}$

0 Mg$^{2+}$
1.7 mM Mg$^{2+}$
3 mM SPM
pH 5.5

n.s.

* * *

n.s.
Figure 8

WT CiVSP

A

B

C

D

trans-membrane helices

Ser 1107

free energy (kJ)

*free energy (kJ)

WT TRPM7  
I100 ramps/Imax

residue at 1107

Electrostatic Component 
Non Bonded Component

 WT phenolype  Mutant phenolype

A S C T D Q E K R

Δ2

Δ3

total

Electrostatic Component 
Non Bonded Component

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