Extracellular Acid Block and Acid-enhanced Inactivation of the Ca\textsuperscript{2+}-activated Cation Channel TRPM5 Involves Residues in the S3-S4 and S5-S6 Extracellular Domains

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Running Title: Acid block of TRPM5

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TRPM5, a member of the super family of transient receptor potential (TRP) ion channels, is essential for the detection of bitter, sweet and amino acid tastes. In heterologous cell types it forms a nonselective cation channel that is activated by intracellular Ca\textsuperscript{2+}. TRPM5 is likely to be part of the taste transduction cascade and regulators of TRPM5 are likely to affect taste sensation. In this report we show that TRPM5, but not the related channel TRPM4b, is potently blocked by extracellular acidification. External acidification has two effects, a fast reversible block of the current (IC\textsubscript{50} pH=6.2) and a slower irreversible enhancement of current inactivation. Mutation of a single Glu residue in the S3-S4 linker and a His residue in the pore region each reduced sensitivity of TRPM5 currents to fast acid block (IC\textsubscript{50} pH = 5.8 for both) and the double mutant was nearly insensitive to acid pH (IC\textsubscript{50} pH = 5.0). Prolonged exposure to acid pH enhanced inactivation of TRPM5 currents and mutant channels that were less sensitive to acid block were also less sensitive to acid-enhanced inactivation, suggesting an intimate association between the two processes. These processes are, however, distinct as the pore mutant H896N, which has normal sensitivity to acid block, shows significant recovery from acid-enhanced inactivation. These data show that extracellular acidification acts through specific residues on TRPM5 to block conduction through two distinct but related mechanisms and suggest a possible interaction between extracellular pH and activation and adaptation of bitter, sweet and amino acid taste transduction.

All animals have evolved mechanisms by which they sense chemicals in the environment and discriminate the nutritious from the noxious. In mammals, taste cells signal the presence of at least five distinct types of chemicals which give us the perceptions of bitter, sour, sweet, salty and amino acid tastes. Two of these modalities, salty and sour, detect ions, Na\textsuperscript{+} and H\textsuperscript{+} respectively, and both modalities may be mediated directly by ion channels on the apical surface of taste cells (1). The other three modalities are mediated by distinct G protein-coupled receptors (2-4) that signal through a common transduction cascade, for which both phospholipase C β2 (PLCβ2)\textsuperscript{1} and the ion channel TRPM5 are essential elements (5). It is likely that upon binding of taste molecules, sweet, bitter and amino acid taste receptors initiate a signaling cascade that

\textsuperscript{1} The abbreviations used are: PLCβ2, phospholipase C β2; PIP\textsubscript{2}, phosphatidylinositol 4,5-bisphosphate; IP\textsubscript{3}, inositol trisphosphate
activates PLCβ2, leading to the breakdown of phosphatidylinositol 4,5-bisphosphate (PIP2) into inositol trisphosphate (IP3) and diacylglycerol (DAG), and the release of Ca2+ from intracellular stores (6, 7). TRPM5 may be the final element of this cascade, converting the biochemical signal transduction pathway into an electrical signal sent to the brain (5, 8). The essential role of TRPM5 in multiple taste pathways suggests that chemicals that modify its activity could have profound effects on taste sensation.

TRPM5 belongs to the TRP family of ion channels, of which there are six sub families: TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPA (ankyrin), TRPML (mucolipin), TRPP (polycystin) (9). Most members of the family are predicted to have six membrane spanning domains and to assemble as tetramers, by analogy to voltage-gated K+ channels (10, 11). The TRPM subfamily contains ion channels that are activated by cold (TRPM8) (12, 13) or ADP-ribose (TRPM2) (14, 15) or that are constitutively active (TRPM1, TRPM3, TRPM6, TRPM7) (9, 16). Only TRPM5 and TRPM4 are activated by Ca2+ (17-21) and both channels likely underlie the Ca2+-activated cation currents observed in a variety of native tissues (e.g. see (22, 23)). The robust activation of TRPM5 by intracellular Ca2+ has allowed careful study of its functional properties. In excised patch recording, TRPM5 channels are activation by Ca2+ with an EC50 of 20 µM and then undergo a process of Ca2+-dependent desensitization, which is partially reversed by the membrane phospholipid PIP2 (20). In whole cell recording, TRPM5 currents are activated by micromolar concentrations of Ca2+ and show pronounced outward rectification, which is due to voltage-dependent gating of the channels (18, 20). TRPM5 currents are selective for monovalent cations and impermeable to divalent cations (18-20). Neither divalent nor trivalent cations block TRPM5 currents.

In this report we show that short exposure to extracellular acidification causes potent and reversible block of mouse TRPM5 and longer exposure inactivates the channels. Proton block is conserved among human and mouse TRPM5 but is not observed for human TRPM4b. We identify critical residues that differ between TRPM4b and TRPM5 that mediate this block.

Materials and Methods

Constructs and mutations- The N-terminus fusion construct of eGFP with mTRPM5 was previously described (20). An eGFP N-terminal fusion to human TRPM5 was kindly provided by D. Prawitt (19). An eGFP hTRPM4b fusion was constructed by cloning a Pmel fragment containing the entire coding sequence of hTRPM4b gene into pcDNA3.1 and inserting a PCR fragment of eGFP at a NotI site immediately upstream of the hTRPM4b coding sequence. Note that while eGFP has a perfect Kozak consensus sequence, it is possible that unfused hTRPM4b could be generated if the internal Kozak sequence on hTRPM4 were to be used. This does not affect the interpretation of our results. MTRPM4b fused to CFP was generated by introducing a Sall site at the initiation codon of mTRPM4b and subcloning Sall –XhoI and XhoI-XbaI fragments of mTRPM4b into eCFP C2 (Clontech). Sequences of fusion proteins were verified (Retrogen) and are available upon request. Point mutations were generated by Quick change mutagenesis (Stratagene) and were verified by sequencing (Retrogen). All constructs were transfected into HEK 293 cells using Fugene (Roche) or Effectene (Quiagen) as described in Liu and Liman (2003) (20). Recordings were performed 24-72 hours after transfection at room temperature.

Electrophysiology-Prior to recording, cells were treated with trypsin (0.05%) EDTA (4 mM) in PBS for 5 minutes at 37°C, followed by gentle trituration to lift adherent cells. This reaction was stopped by the addition of media containing 10% FBS. Cells were used for up to 8 hrs after enzyme treatment. Immediately prior to recording, cells were plated in the recording chamber. After formation of a gigaohm seal, cells were moved in front of a linear array of micro perfusion pipes under computer control from which test solutions were delivered (Warner Instruments) and whole cell recording was initiated. For all experiments, the membrane potential was held at –80 mV.

Standard intracellular solution was (in mM): CsGlutamate 125, NaCl 8, MgATP 2, HEPES 10, HEDTA 2, CaCl2 1.5 (40 µM free Ca2+; pH 7.2). High Ca2+ intracellular solution was (in mM): CsGlutamate 125, HEPES 10, CaCl2 0.5 (pH 7.2). Junction potentials for these 2 solutions of 13 and 18 mV respectively were corrected in plotting I-V
relations. External solutions of varying pH contained (in mM): NaCl 140, CaCl$_2$ 2, MgCl$_2$ 1, adjusted with HEPES 10 (pH 8.0, 7.1, 6.8), or MES 10 (pH 6.8, 6.5, 6.3, 5.9, 5.4, 5.0, 4.0), or TAPS 10 (pH 8.5, 8.0). Note that we compared the magnitude of TRPM5 currents in solutions buffered to pH 8.0 or 8.5 with HEPES, TAPS and AMPSO and observed no difference in current amplitudes, indicating that the buffers themselves did not appear to affect the TRPM5 currents. We also found no evidence for block of the current by MES (see Fig. 1). 10 mM citric acid solutions contained (in mM): NaCl 130, citric acid 10, CaCl$_2$ 2, MgCl$_2$ 1, MES 10 (pH 6.3 or 5.9). 100mM citric acid solutions contained (in mM) NaCl 10, citric acid 100, CaCl$_2$ 2, MgCl$_2$ 1, MES 10 (pH 6.3 or 5.9).

A standard dose-response protocol was used to test for acid block and is depicted in Fig. 1B. The membrane potential was ramped from –80 - +80 mV (160 mV/180 ms) at 2 Hz. To test for block, the control external solution (pH 7.1 or 8.0) was exchanged for an acidic external solution for 2 s, followed by a 2-6 s recovery at the neutral pH. For most experiments, a 2 s recovery period in the control solution was sufficient, as judged by the fact that the currents returned to steady level (see Fig. 1B). In some cases we lengthened the recovery period to be sure that recovery had reached steady state. To quantitate the amount of block, we measured the magnitude of the current at the end of the 2 s of exposure to the test solution (at +80 mV) and comparing this to the magnitude of the current following recovery (at the end of 2 s of recovery, unless otherwise stated). This experimental design provides a measure of the fraction of current that was reversibly blocked by protons. There was no difference in the results when the control solution was pH 7.1 or pH 8.0 and these data were therefore combined.

To measure the rate of inactivation of TRPM5 currents, we measured currents in response to ramp depolarizations (-80 - +80 mV; 1V/s) applied every s, starting immediately upon break in to the whole-cell configuration. For some experiments, the external solution was exchanged prior to recording (Fig. 6) while in others (Fig. 7) the external solution was exchanged during the recording.

Patch pipets (1.5-3 mOhm) were fabricated from borosilicate glass. All recording were made with an Axopatch 200B amplifier, digitized with a digidata 1322a and acquired with pClamp 8.2 or 9.0. Records were sampled at 5 kHz and filtered at 1 kHz. Data was analyzed with Clampfit 8.2 or 9.0 (Axon Instruments) or Origin 6.1 (Originlab Corp.). Dose-response curves were fit with a Hill equation of the form: \( y = \frac{V_{\text{max}} \cdot (x^n)}{(IC_{50} - x^n)} \). All data are presented as mean ± s.e.m. Data was analyzed with a student’s t-test (1-tailed or 2-tailed as appropriate).

Results

TRPM5 is blocked by extracellular acidification

When expressed in HEK 293 cells, mouse TRPM5 (mTRPM5) forms a cation selective conductance that is activated by elevation in intracellular Ca$^{2+}$. Activation of mTRPM5 is easily detected by voltage ramps (-80 to +80 mV), which elicit a characteristic outwardly rectifying current in mTRPM5 expressing cells that are dialyzed with 40 µM Ca$^{2+}$ (Fig. 1) (18-20). To allow identification of cells expressing TRPM5, we used a fusion protein of eGFP to the N terminus of mouse TRPM5, which we will henceforth refer to as simply mTRPM5. Confocal microscopy reveals that this fusion protein localized at or near the cell membrane (Fig. 1A). In previous experiments we found that this channel behaves similarly to the unmodified channel (20). All experiments were conducted in HEK 293 cells, which showed virtually no endogenous currents under the conditions of this study (20).

To determine if mTRPM5 currents are sensitive to changes in external pH we exposed HEK cells expressing mTRPM5 to external solutions at varying pH. External acidification (pH 5.4) produced a rapid block of the currents (Fig. 1B), which recovered upon return to neutral pH. Block at acid pH was observed regardless of whether MES or HEPES was used as buffer, indicating that it was not due to block by the pH buffer itself (Fig. 1D). Proton block of mTRPM5 currents was dose-dependent and could be fit with a Hill equation with half block (IC$_{50}$) at pH 6.2 (8 cells, Fig. 1C,D). A Hill coefficient (n$_h$) of 3.0 was obtained from the fit to these data, suggesting that multiple identical or nonidentical sites on the channel are protonated.

mTRPM5 currents are sensitive to voltage and acid block of mTRPM5 currents could therefore result from a shift in the voltage dependence of activation, such that larger depolarizations are necessary to open the channels a low pH. If this were the case, we expected that the pH sensitivity of the currents at low voltages might be greater than at
high voltages. Instead we observed similar block of mTRPM5 currents at –80 mV (IC$_{50}$ = 6.4, nh = 2.3, 8 cells; Fig. 1D) as we did at +80 mV. A more comprehensive study of the effects of pH on the activation of mTRPM5 currents was complicated by time-dependent shifts in the activation of these currents (D. Liu and E.R. Liman, unpublished).

If the sensitivity of mTRPM5 to pH serves a functional purpose, we reasoned that it should be conserved across species. Mouse TRPM5 and human TRPM5 (hTRPM5) are ~85% identical at the amino acid level. A GFP hTRPM5 fusion protein (19) was transfected into HEK 293 cells and currents were induced by intracellular dialysis of 40 µM Ca$^{2+}$. This resulted in the induction of large outwardly rectifying currents in cells expressing hTRPM5 (Fig. 2A, $I = 1.91 +/- 0.26$ nA at +80, n = 11) that rapidly inactivated. Note that our results appear to be in conflict with data from Prawitt et al. (19) who recorded from cells stably expressing this channel and found that currents were maximally activated by 500 nM Ca$^{2+}$ and potently inhibited by micromolar concentrations of Ca$^{2+}$. Despite the rapid rundown of hTRPM5 currents, in a few cells the current amplitudes stabilized and in these cells exposure to solutions of pH 6.5 or lower potently blocked the current (Fig. 2B,C; at +80 mV, fractional remaining current after a 2s exposure to pH 6.5 was 0.07 +/- 0.03 of the current immediately prior, n = 4). Recovery from block was generally not observed and we attribute this to an enhancement of inactivation at low pH, as described later. The block of hTRPM5 by protons is an evolutionarily conserved feature of the channel and is likely to be of functional significance for mice and humans.

**Sensitivity to pH is a specific feature of TRPM5**

To understand whether proton sensitivity is a specific feature of TRPM5, we determined if the related ion channel TRPM4 is also sensitive to external pH. Human TRPM4b (hTRPM4b) was fused to GFP and transfected into HEK 293 cells. To induce hTRPM4b currents, we dialyzed transfected cells with 0.5 mM Ca$^{2+}$. Note that while an earlier report found a relatively high sensitivity of hTRPM4b to Ca$^{2+}$ (17), more recent data indicates that this channel has a low sensitivity to Ca$^{2+}$ (IC$_{50}$ = 0.37 mM) (21). Under these conditions, large outwardly rectifying currents were induced in hTRPM4b expressing cells that were not observed in untransfected cells (Fig. 2D; $I = 1257 +/- 250$ pA at +80 mV, n = 9 in TRPM4b cells; $I = 102.3 +/- 16.4$ pA, n = 9 in untransfected cells). In contrast to mouse and human TRPM5 currents, hTRPM4b currents showed no sensitivity to external pH (Fig. 2E,F), and lowering the pH to 5.4 produced virtually no block of the currents (at +80 mV, fraction of current remaining at pH 5.4 = 0.89 +/- 0.02, n = 7).

To determine whether the high concentration of Ca$^{2+}$ used to induce these currents might affect the pH sensitivity of the channels, we induced mTRPM5 currents under the identical conditions. mTRPM5 channels remained sensitive to pH with nearly complete block of the currents at pH 5.4 (fraction of current remaining at pH 5.4 = 0.13 +/- 0.07, n = 5).

**Effect of extracellular pH is not mediated by intracellular acidification**

The rapid and reversible block of mTRPM5 currents at low external pH suggested that block of the channel is due to protonation of an external binding site. This is likely because in all experiments the intracellular solution of the cell was buffered to pH 7.4 with 10 mM HEPES. However, it is possible that lowering the external pH could have induced a change in the internal pH of the cell close to the ion channel. Therefore, we tested the pH sensitivity of mTRPM5 currents when the pH of the intracellular solution was heavily buffered with 100 mM HEPES in the pipette. No difference was observed as compared with weaker pH buffering (Fig. 3A; IC$_{50}$ = pH 6.1, nh = 3.2), supporting the view that intracellular acidification does not play a role in the block by low external pH that we observe.

To further test whether the proton block that we observe is due to intra or extracellular acidification, we compared the sensitivity of mTRPM5 currents to proton block in the presence of a weak acid, citric acid or a strong acid, HCl. Weak acids can exist in a undissociated form that can cross cell membranes and thereby acidify intracellular compartments of cells. Indeed this is believed to be the mechanism for sour taste transduction (24). Fig. 3 shows that mTRPM5 currents were similarly sensitive to external solutions of the same pH value, regardless of whether Na citrate/citric acid (10 mM or 100 mM replacing NaCl) or NaCl/HCl was used to prepare the solution (Fig. 3B,C). These data lead us to conclude that block of mTRPM5 by low pH
solution under these conditions is not mediated by internal acidification.

Identification of a residue in the pore region of TRPM5 that mediates acid block

Based on the preceding experiments, it is likely that external protons block mTRPM5 channels through the protonation of one or more extracellular amino acid residues. We therefore systematically mutated residues that were likely to be extracellular and could be protonated over the range of pH values for which we observe block of mTRPM5 currents. Note that the predicted pKₐs of amino acids that we mutated are: His = 6.0, Glu = 4.3, Asp = 3.7. We mutated Glu and Asp because it had been previously shown that the pKₐs of these amino acids can be shifted to ~pH 7 through the coordination of a single proton by neighboring carboxylate side chains (25).

The S5-S6 linker of mTRPM5 is likely to be extracellular and may form the pore-lining region, by homology to the structure of voltage-gated K⁺ channels (26, 27). Residues in this region have been shown to mediate proton sensitivity in other channels, including the distantly related capsaicin receptor, TRPV1 (28). Within the S5-S6 linker of mTRPM5, 9 residues were singly or doubly mutated (Fig. 4A) and five of the mutants formed functional channels that could be activated in response to intracellular dialysis of 40 µM Ca²⁺. The reversal potential of currents generated by all mutants under the bi-ionic conditions used was close to 0 mV, indicating that none of the mutations significantly changed the monovalent selectivity of the channel (Fig. 4D and data not shown). Sensitivity to proton block was assessed for each mutant using a standard dose-response protocol and summary information for all mutants is shown in Fig. 4B and Table 1. One mutation, H934N, dramatically reduced the sensitivity of the channels to external acidification (Fig. 4B,C). The dose-response relation for H934N is fit with a midpoint of pH 5.8, which indicates an ~3 fold lower sensitivity to protons as compared with wild-type channels (midpoint of pH 6.2). Interestingly the dose-response relationship is also less steep in this mutant; the Hill coefficient is 1.8 as compared with 3.0 for wild-type channels. The Hill coefficient of 3 for wild-type channels suggests the presence of at least four proton binding sites on the presumed tetrameric channel all of which need to be occupied to block conduction. It is unlikely that these four sites are contributed by the single residue that we identified as the charge-neutralizing mutant H934N still retained significant pH sensitivity. Instead, we favor the interpretation that there are two distinct types of sites, perhaps formed by dimers of subunits (25), both of which need to be protonated (or neutralized) to block channel conduction. Under this interpretation, neutralization of a single site, such as H934, should reduce the Hill coefficient by half, and another site should contribute to the residual proton sensitivity.

Identification of a residue in the S3-S4 linker region of TRPM5 that mediates acid block

Because H934N currents showed residual pH sensitivity, we examined the sequences of the S1-S2 and S3-S4 linkers for additional residues that were titratable near neutral pH and might mediate proton block. Mutations of His, Glu and Asp residues in the S1-S2 linker did not alter sensitivity of the channels to pH (see Table 1). Note that these mutations were made as double mutants in the background of the H934N mutant since we expected this would make improve our ability to detect a subtle change in pH sensitivity conferred by a second site. In the S3-S4 linker there is just one protonatable residue (E830, Fig. 5A). Mutation of this residue to a neutral residue (E830Q) produced channels that were considerably less sensitive to external protons (IC₅₀ pH=5.8, nₕ=1.4), suggesting that this site also plays a role in mediating proton block of the channels (Fig. 5B, C; Table 1). Moreover, the double mutant of E830Q with H934N was nearly insensitive to external pH, indicating that these 2 residues account for most of the proton sensitivity of mTRPM5 (Fig. 5B, D; IC₅₀ pH = 5.0). Interestingly, for the double mutant, the Hill coefficient was further reduced to a value of 0.5, consistent with the loss of even more proton binding sites in this mutant as compared with the single mutants.

To gain insight into the mechanism by which E830 influences the sensitivity of mTRPM5 channels to proton block we compared the effects of charge conserving and charge neutralizing mutations at this position. If the negative charge at position 830 plays a simple electrostatic role in regulating conduction through the channel (either by influencing gating or permeation), then charge neutralizing mutations should have the same phenotype as lowering pH – that is a dramatic reduction in current. This was clearly not the case, as
both E830Q and E830N mutants generated large currents (3.00 ± 0.31 nA, n = 22 and 2.52 ± 0.30 nA, n = 17 respectively). Moreover, a simple electrostatic model predicts that the E830D channel should be similarly sensitive to pH as the wild-type channel. Instead we observed that the E830D channel was less sensitive to pH than the wild-type channel and that a double mutant channel (E830D/H934N) was less sensitive to pH than a single mutant of H934N (Fig. 5 E,F). These results are not consistent with the hypothesis that 830E plays a purely electrostatic role in governing conduction through mTRPM5. Rather, they suggest that 830E plays an allosteric role, in which it interacts with other residues in the channel protein and regulates the open conformation of the channel.

Mouse TRPM4b (mTRPM4b) differs from hTRPMb in having an aspartic acid residue instead of a histidine at the equivalent to position 830E. However, like hTRPM4b, mTRPM4b was completely insensitive to acidic pH (at +80 mV, the fraction of current remaining at pH 5.4 = 1.05 ± 0.02, n = 5). These data indicate that the presence of an acidic residue at position 830 is not sufficient to produce acid block of TRPM4 and are consistent with our data suggesting that 830E plays an allosteric role in regulating ion conduction through the channel.

Long exposure to low extracellular pH enhances inactivation of TRPM5 currents

While doing these experiments, we noticed that current recovery following exposure to acidic pH was often not complete. Previously we found that following activation in whole cell or perforated patch recording mode, mTRPM5 currents inactivate (20). We wondered whether acid pH enhanced the inactivation of mTRPM5 currents. To test this, we measured the time course of inactivation of whole cell currents in cells bathed in external saline at pH 6.5, which blocks <50% of the current, with those bathed in saline at neutral pH (7.1). For all experiments, cells were bathed in the appropriate solution for >10 s prior to whole cell recording, so that the fast proton-induced block of the channels should have reached equilibrium. Bathing cells in external solution at pH 6.5 significantly enhanced the extent of inactivation of the currents, without significantly affecting the rate of inactivation (Fig. 6A). For example, 60 seconds following the peak of the current, 89 ± 3% (n = 9) of the current was inactivated for cells bathed in solution of pH 6.5, as compared with 51 ± 5% (n = 6) of the current for cells bathed at pH 7.1 (P < 0.001). Because the decay of the currents did not follow a simple exponential time course, we quantified the rate of decay by measuring the time at which the current had undergone 50% of its decay. This was 9.5 ± 1.9 s for cells bathed at pH 6.5 and 13.6 ± 2.5 s for cells bathed at pH 7.1 (P > 0.2). Thus low pH appears to increase the fraction of current that is inactivated but not the rate of inactivation.

We next asked whether the same residues that mediate acid block of mTRPM5, also mediate acid-enhanced inactivation. We showed previously that currents generated by the pore mutant H934N were relatively insensitive to a 2 s exposure to pH 6.5 (see Fig. 4). We therefore tested whether exposure to pH 6.5 affected inactivation of H934N currents and found no significant increase in the amount of inactivation measured 60 s after the peak of the current (Fig. 6, P > 0.8). Similarly, currents from the S3-S4 mutant E830N were not blocked by a short exposure to pH 6.5 (see Fig. 5) and prolonged exposure to pH 6.5 did not enhance the amount of inactivation of E830N currents (Fig. 6, P > 0.7). On the other hand, we previously found that currents generated by both mutants were ~50% blocked at pH 5.9 (Fig. 4,5), and at this pH, the amount of inactivation was significantly enhanced for both mutants (Fig. 6, P < 0.001 for both as compared with pH 7.1). Finally we tested the double mutant E830N/H934N, which was not blocked by a short exposure to pH 5.9 and was ~50% blocked by pH 5.0. In accordance with our previous results, this mutant only showed enhanced inactivation at pH 5.0 and not at pH 5.9 (Fig. 6, P < 0.001 and P > 0.8, respectively). Thus acid-enhanced inactivation appears to be intimately associated with acid block of the channels.

In these experiments, we functionally defined inactivation as a state that channels enter following Ca2+ activation from which they do not open, and cannot be recovered. Therefore to determine if indeed low pH induces inactivation of mTRPM5 channels, we tested whether currents that decayed during prolonged exposure to acid pH could be recovered at neutral pH. As seen in
Fig. 7A,C, following a 100s exposure of the currents to pH 6.5, no current could be recovered even with a long incubation at pH 7.1. Thus prolonged exposure to acid pH does induce inactivation of mTRPM5 channels.

In the process of testing mutants for acid block, we found one residue, 896H, which appeared to play a role in regulating acid-induced inactivation. The H896N mutant had a normal sensitivity to fast acid block (see table 1), but recovered nearly completely from a short exposure to low pH (data not shown). We therefore tested whether currents generated by this mutant could recover from acid-induced inactivation. As seen in Fig. 7B,C, exposure to pH 6.5 induced inactivation of H896N currents to a similar extent as wild-type currents, but upon return to neutral pH, there was a significant recovery of the current. This recovery took tens of seconds and thus is not due to the fast unblock of the channel that we observed following short exposure to this pH. Further, this data shows that acid-enhanced rundown of mTRPM5 is likely due to entry of the channels into an inactivated state from which H896N, but not wild-type channels can recover.

Human TRPM5 showed even more pronounced acid-induced inactivation, which could be seen even with a 2 s exposure to acid pH (Fig. 8A and Fig. 2). We therefore mutated the residue equivalent to 896H in the human clone (898H) and determined whether this mutation affected acid-induced inactivation. H898N hTRPM5 currents, although blocked to a similar degree as wild-type currents by pH 6.5, recovered significantly more completely (Fig. 8B,C). To determine if this was due simply to a slowing of the rate of inactivation of the mutant as compared to the wild-type channel, we measured the rate and extent of inactivation of wild-type and mutant channels at neutral pH. When measured in external solution of pH 7.4, the wild-type hTRPM5 currents had a peak amplitude of 1.26 ± 0.28 nA, half inactivated in 11.6 ± 1.9 s with a residual current of 10.5 ± 1.5 % at 60 seconds (n = 11). H898N currents did not differ significantly from wild-type currents and had a peak amplitude of 1.47 ± 0.43 nA, half inactivated in 14 ± 2.5 s with a residual current of 10.4 ± 1.7 % at 60 seconds (n = 12). Thus we have identified a residue that appears to play a specific role in recovery from acid-induced inactivation of TRPM5 currents. These experiments further confirm that acid-induced inactivation is molecular distinct from, although associated with, acid block of TRPM5.

Discussion

In this report we show that TRPM5, a Ca²⁺-activated cation channel that mediates taste transduction and is expressed in intestine and stomach, but not the related channel TRPM4b, is potently blocked by extracellular acidification. External acidification has two effects, a fast reversible block of the channels and a slower irreversible inactivation of the currents. We identify two critical amino acid residues that determine sensitivity to fast acid block, which are located in the proposed pore and S3-S4 linker of the channel (Fig.9). Mutations of either of these two residues reduces proton sensitivity by 3-fold and mutation of both reduces proton sensitivity by >10-fold. In addition, we identify a single residue in the pore region that regulates acid-induced inactivation of the currents (Fig. 9). These three residues are conserved in human and mouse TRPM5 but not in TRPM4b, suggesting that they play an evolutionarily conserved role in regulating activity of TRPM5.

TRPM5 is prominently expressed in taste cells and in the gut (8), and thus channels are likely to be exposed to acidic pH under physiological conditions. Taste cells are bathed in saliva which under ambient conditions, has a pH of 6-7, but can be further acidified by the ingestion of sour-tasting foods which can have a pH as low as 3 (24). At present the mechanism of sour transduction is not well understood and several candidate pH-sensitive ion channels have been proposed to mediate this modality (24). Our observation that TRPM5 is pH sensitive makes it a candidate to play a role in sour taste transduction. However, it is unlikely that it is a central component of sour transduction as targeted deletion of TRPM5 in mice does not appear to impair detection of sour tastes (5). Moreover, we found that there was no difference in the block of TRPM5 by solutions that contained a weak acid, citric acid, as compared to those that contained a strong acid, HCl, although the former is considerably more sour tasting (24). We therefore consider it more likely that acidic pH modulates sensory response mediated by TRPM5, such as the responses to bitter, sweet or amino acid tastes.
Although one study found no interaction between pH and sweet taste in humans (29), this experiment did not directly address the possibility of interaction at the sensory receptor level which could be compensated by central mechanisms. Alternatively, it is possible that in taste cells TRPM5 assembles as a multimer with other subunits such that the assembled channel is not pH sensitive. In this case, proton block of TRPM5 might serve to functionally eliminate misassembled channels. Finally, it is possible that TRPM5 channels are not present on the apical surface of the taste cells, and therefore not exposed to varying extracellular pH. Proton block might serve to functionally eliminate mislocalized channels in this case.

Extracellular acidification has been shown to regulate the activity of a large number of ion channels, including several members of the TRPV subfamily of ion channels. Extracellular acidification potentiates TRPV1 (30), which is activated by heat and capsaicin (31), while it blocks the related channel TRPV5 (32). Potentiation of TRPV1 by protons may play a role in inflammatory hypersensitivity of pain responses (33), while block of TRPV5 by protons may play a role in regulating Ca\textsuperscript{2+} uptake by the kidney (32). Proton effects on these two channels map to extracellular Glu residues in the linker between the S5 region and the proposed pore region of the channel (28, 32). Our results show that proton block of mTRPM5 is instead mediated by a His residue (934\textsuperscript{H}) that lies between the proposed pore region and the S6 transmembrane domain and a Glu residue (830\textsuperscript{E}) in the S3-S4 linker. Thus the mechanism of acid block of TRPM5 is likely to be different from that of TRPV5. Instead, the location of residues we identified are remarkably similar to the location of residues involved in acid activation of a K\textsuperscript{+} channel from plants (34), suggesting a possible conservation in mechanisms of proton regulation between TRP channels and distantly related K\textsuperscript{+} channels. From this perspective, it is interesting to note that in K\textsuperscript{+} channels, the S3-S4 linker and the pore region have been shown to be in close proximity to each other (35). Our observation that mutations in both the S3-S4 linker and pore region of mTRPM5 have similar effects on proton block of the channel suggests that in TRP channels these regions may also be closely apposed.

Our data also suggest a possible conservation in the mechanism of inactivation of TRPM5 and some voltage-gated K\textsuperscript{+} channels. K\textsuperscript{+} channels in the Kv family can undergo two distinct types of inactivation: N-type, which involves interaction of the N terminus of the channel with the cytoplasmic vestibule of the open channel (36) and C-type, which is regulated by residues in the pore region and S6 of K\textsuperscript{+} channels and involves a global change in channel conformation (37). In some Kv channels, the rate of C-type inactivation increases at acid pH (38, 39), and this effect is mediated by a His residue (896\textsuperscript{H} in Kv1.5) between the S5 and pore region of the channel. We found that acid pH enhances inactivation of mTRPM5 currents, and a His residue in the corresponding region of TRPM5 plays a critical role in this process. Entry into the inactivated state is more complete from the proton-blocked state than from the unblocked state and mutations that affect the IC\textsubscript{50} for proton block similarly affect acid-enhancement of inactivation. In the mutant H896N, channels can recover from acid-induced inactivation, suggesting that protonation of this residue may stabilize the inactivated state of the channel. Together these data can be interpreted to suggest that acid-enhanced inactivation of TRPM5, and perhaps inactivation at neutral pH as well, involves re-arrangements of the pore region of the channel in a manner similar to the current model for C-type inactivation of K\textsuperscript{+} channels.

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Figure Legends:

Figure 1. mTRPM5 is blocked by low external pH.  A, Visualization of GFP::mTRPM5 in a transfected HEK 293 cell by confocal microscopy shows distinct membrane localization of the fusion protein. RFP was co-transfected to allow visualization of the intracellular compartments of the cell. B, mTRPM5 currents in a HEK 293 cells elicited by intracellular dialysis with 40 μM Ca²⁺ through the patch pipette in response to voltage ramps (-80 mV to +80 mV, 160 mV/180 ms) in varying external solution. Note that changing the external solution from pH 8.5 to pH of 5.4 led to a complete block of the current, which was largely reversible. C, Currents in response to voltage ramps (as in B) at varying external pH levels in an mTRPM5 expressing cell. D, Average dose-response data for proton block of mTRPM5 currents measured at the voltages indicated. A single data point is shown for block of the currents at an acidic pH (6.0) using 10 mM HEPES as buffer.

Figure 2. hTRPM5 is sensitive but hTRPM4b is not sensitive to acid pH.  A,D. Time course of currents elicited in a HEK 293 cell transfected with either hTRPM or hTRPM4b, induced by intracellular dialysis with 40 μM Ca²⁺ (hTRPM5) or 0.5 mM Ca²⁺ (hTRPM4b). Currents were elicited in response to voltage ramps (-80 mV to +80 mV, 160 mV/180 ms) from a holding potential of –80 mV. B,E. Changing the pH of the external solution did not alter the magnitude of TRPM4b currents but potently, and irreversibly blocked hTRPM5 currents. Currents were elicited in response to voltage ramp (-80 mV to +80 mV, 160 mV/180 ms) from a holding potential of –80 mV. Control solution was pH 8.0 for hTRPM5 and pH 7.1 for hTRPM4b. C, F, I-V relation for hTRPM5 currents from B and for TRPM4b currents from E.

Figure 3. Block of mTRPM5 is mediated by extracellular protons. A. Similar pH sensitivity of TRPM5 currents with intracellular pH buffer of 10 mM and 100 mM HEPES. Dose-response data was obtained at +80 mV as in Figure 1. B. Block of TRPM5 currents by low pH in the presence of a weak acid (citric acid) at 10 mM or 100 mM in the extracellular solution. Note that there is no increase in the block in the presence of citric acid. C. Average data from B for 10 mM citric acid (top) and 100 mM citric acid (bottom). There was no enhancement of block with citric acid as compared to control solution. CA, citric acid.

Figure 4. Proton block is partly mediated by a residue in the pore region of TRPM5.  A. Sequence alignment of the proposed pore region of mTRPM5 with that of hTRPM4. Residues that were mutated in TRPM5 are indicated. B. Fraction of current that was insensitive to block at two pH levels for wild-type and pore region mutant channels. Mutants were tested as in figure 1 and Currents were measured at +80 mV. Mutants that did not form functional channels could not be characterized. Data from each mutant was compared with the data from wild-type channels using a one-tailed t-test. ** P < 0.001. C. Dose-response relation for wild-type and H934N currents at + 80 mV in response to varying external pH. D. H934N currents in response to ramp depolarizations with varying external pH. Note that the rectification and reversal potential of the mutant currents were indistinguishable from those of wild-type currents.

Figure 5. A residue in the S3-S4 linker contributes to proton block.  A. Sequence alignment of the S3-S4 linker of mTRPM5 with that of hTRPM4, showing the position of the mutant studied. B. Dose-response relation for wild-type and E830Q and E830Q/H934N currents in response to varying pH. Dose- response relation for wild-types currents (dashed) is re-plotted from Figure 1. Note that the double mutant is relatively insensitive to pH. C,D E830Q and E830Q/H934N currents in response to ramp depolarizations with varying external pH. Note that the rectification and reversal potential of the mutant currents were indistinguishable from those of wild-type currents E. Dose-response relation to varying pH for 830E mutant channels with different amino acid substitutions. F, Dose-response relation to varying pH for double mutants of H934N and 830E mutant channels with different amino acid substitutions. Dose-response relation for wild-types currents (dashed) is re-plotted from Figure 1.
Figure 6. Acid-enhanced inactivation of TRPM5 is linked to acid block. A. Time course of current decay of whole cell currents from wild-type mTRPM5 at pH 7.1 and pH 6.5. Currents in HEK cells were induced by interacellar dialysis with 40 μM Ca\(^{2+}\) and ramp depolarizations (-80 to +80 mV) were applied at 1 Hz. Current at +80 mV was normalized to the peak current and averaged for 5-6 cells. Data is shown ± S.E.M. Note the increased rate of inactivation of the currents at pH 6.5. B, C, D, Time course of current inactivation for mutants E830Q, H934N and E830Q/H934N at pH 7.1-pH 5.0, obtained as in A. Note that currents conducted by mutant channels are less sensitive to acid-enhanced inactivation. Black circle, pH 7.1, yellow triangle, pH 6.5, blue triangle, pH 5.9, magenta square, pH 5.0. ** indicates that the residual current 60 s after the peak current was significantly reduced in this condition as compared with pH 7.1 (student’s t-test).

Figure 7. A residue in the pore of TRPM5 regulates acid-enhanced inactivation in mTRPM5. A, B. Whole cell currents in HEK cells expressing wild-type or H896N mTRPM5 in response to ramp depolarizations (-80 mV to +80 mV; 1V/s, applied every second). Following inactivation at pH 6.5, wild-type TRPM5 currents cannot be recovered at pH 7.1, whereas H896N currents partially recover. Filled circles are current at +80 mV, open circles are current at -80 mV. C. Magnitude of wild-type and H896N currents following acid-enhanced inactivation at pH 6.5 and recovery at pH 7.1. Data was obtained from experiments like those in A, B and magnitude of currents at +80 mV were measured after 100 s at pH 6.5 or 100 s recovery in pH 7.1. Note that H896N currents inactivate to a similar degree as wild-type currents but significantly more current is recovered at pH 7.1.

Figure 8. The same residue regulates recovery from proton block of hTRPM5. A, B. Whole cell currents in HEK cells expressing wild-type or H898N hTRPM5 in response to ramp depolarizations in varying external solution. Cells were held at -80 mV and voltage ramps (-80 - 80 mV) were applied as in Figure 1. A. Following a 2 s exposure to pH 6.5, wild-type currents show only partial recovery from block, suggesting that inactivation of the channels was accelerated at pH 6.5. B. In contrast, H898N hTRPM5 currents are blocked by exposure to pH 6.5, but show nearly complete recovery. F, Summary of D, E, showing the fraction of current recovered after a 2 s exposure to pH 6.5 for wild-type and H898N currents. Fractional current recovery was determined by measuring the current 2 s after return from pH 6.5 to pH 7.1 (when currents have recovered from block) and dividing by the current immediately prior to exposure to pH 6.5. ** P < 0.01, student’s t-test.

Figure 9. Summary of mutants that affect acid block and acid-enhanced inactivation. E830N and H934N each play a role in acid bloc and H896N plays a role in recovery from acid-enhanced inactivation.

Table 1. Sensitivity to fast acid block of wild-type and mutant mTRPM5 channels. Currents were induced in transfected HEK cells by intracellular dialysis with 40 μM Ca\(^{2+}\). Inhibition by acid pH was quantitated by construction of dose-response curves that were fitted with a Hill equation of the form y = V\(_{max}\) (x\(^n\) / (IC\(_{50}\)\(^{n_h}\) + x\(^{n_h}\))) where V\(_{max}\) = 1. IC\(_{50}\) is presented in units of molarity and pH. n\(_h\) is the Hill coefficient. n is the number of cells from which the data was derived.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7

A

B

C

**Figure 7**
Figure 8
Figure 9

- Acid block
- Acid-induced inactivation
| Mutant                  | IC50 [H+] (µM) | pH | n, | n |
|------------------------|----------------|----|----|---|
| WT                     | 0.62±0.03      | 6.2| 3.0±0.3 | 8 |
| Single mutant          |                |    |    |    |
| S3-S4                  |                |    |    |    |
| E830Q                  | 1.74±0.34      | 5.8| 1.4±0.3 | 6 |
| E830D                  | 1.22±0.23      | 5.9| 1.6±0.4 | 7 |
| E830N                  | 1.12±0.25      | 6.0| 1.8±0.6 | 8 |
| S5-S6                  |                |    |    |    |
| H896N                  | 0.73±0.10      | 6.1| 2.5±0.6 | 7 |
| E901A                  | 0.67±0.08      | 6.2| 3.2±0.8 | 6 |
| E926Q                  | 0.67±0.05      | 6.2| 3.7±0.7 | 6 |
| H934N                  | 1.43±0.11      | 5.8| 1.8±0.2 | 7 |
| E939G                  | 0.90±0.14      | 6.0| 3.3±1.2 | 9 |
| Double mutant with H934N|              |    |    |    |
| S1-S2                  |                |    |    |    |
| EDA667QNS              | 1.88±0.14      | 5.7| 1.7±0.2 | 4 |
| DLED674NLQN            | 1.41±0.19      | 5.9| 1.9±0.4 | 8 |
| DLQEPD677EARQPN        | 1.66±0.13      | 5.8| 1.5±0.2 | 8 |
| LDME684INMQ            | 1.38±0.14      | 5.9| 1.8±0.3 | 7 |
| E699Q                  | 1.95±0.12      | 5.7| 1.9±0.2 | 6 |
| E703Q                  | 1.65±0.33      | 5.8| 1.9±0.6 | 5 |
| APGD707GRAN            | 1.93±0.10      | 5.7| 1.8±0.1 | 5 |
| S3-S4                  |                |    |    |    |
| E830Q                  | 10.0±2.3       | 5.0| 0.5±0.1 | 10 |
| E830D                  | 2.42±0.53      | 5.6| 1.0±0.2 | 6 |
| E830N                  | 3.02±0.64      | 5.5| 1.1±0.2 | 6 |

Table 1
Extracellular acid block and acid-enhanced inactivation of the Ca\textsuperscript{2+}-activated cation channel TRPM5 involves residues in the S3-S4 and S5-S6 extracellular domains

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