Potentiation of TRPC5 by Protons

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Mammalian members of the classical transient receptor potential channel subfamily (TRP) are Ca2+-permeable cation channels involved in receptor-mediated increases in intracellular Ca2+. TRPC4 and TRPC5 form a group within the TRPC subfamily and are activated in a phospholipase C-dependent manner by an unidentified messenger. Unlike most other Ca2+-permeable channels, TRPC4 and -5 are potentiated by micromolar concentrations of La3+ and Gd3+. This effect results from an action of the cations at two glutamate residues accessible from the extracellular solution. Here, we show that TRPC4 and -5 respond to changes in extracellular pH. Lowering the pH increased both G protein-activated and spontaneous TRPC5 currents. Both effects were already observed with small reductions in pH (from 7.4 to 7.0) and increased up to pH 6.5. TRPC4 was also potentiated by decreases in pH, whereas TRPC6 was only inhibited, with a pIC50 of 5.7. Mutation of the glutamate residues responsible for lanthanoid sensitivity of TRPC5 (E543Q and E595Q) modified the potentiation of TRPC5 by acid. Further evidence for a similarity in the actions of lanthanoids and H+ on TRPC5 is the reduction in single channel conductance and dramatic increase in channel open probability in the presence of either H+ or Gd3+ that leads to larger integral currents. In conclusion, the high sensitivity of TRPC5 to H+ indicates that, in addition to regulation by phospholipase C and other factors, the channel may act as a sensor of pH that links decreases in extracellular pH to Ca2+ entry and depolarization.

The TRP2 ion channel family consists of over 25 proteins that are homologous to Drosophila TRP. Like many other cation channels, TRP channels are proposed to have six transmembrane segments (S1–S6) with a re-entrant pore-forming loop between S5 and S6, and intracellular C and N termini (for reviews, see Refs. 1–5). In addition to the transmembrane topology, a number of other structural features are shared between subfamilies. Thus, TRPC, TRPV, and TRPM channels have a characteristic sequence, the TRP box (Fig. 1B), not far from S6 in their cytosolic C termini, and often have regulatory calmodulin-binding domains in the C-terminal tail. TRPC, TRPV, and TRPA channels have N-terminal, cytosolic ankyrin-like repeats, the functional role of which is still unclear. TRP channels also share functional features. Where channel activity has been demonstrated, TRP channels have been shown to be cation channels with relative Ca2+-permeabilities (PNa/PCa) ranging from low (TRPM4 and -5) to high (TRPV5 and -6). Many TRP channels are regulated by phospholipase C (PLC)-dependent mechanisms, including members of the TRPC subfamily, which show PLC-dependent activation, and some TRP channels from other subfamilies, which display PLC-dependent modulation or activation.

TRPC4 and TRPC5 are receptor-operated, PLC-dependent, Ca2+-permeable nonselective cation channels. However, in contrast to several other members of the TRPC subfamily that are activated by diacylglycerol, the steps linking PLC activity to channel activation remain unclear (for a review, see Ref. 6). For TRPC5, additional activators have been described, the physiological significance of which is uncertain (7–9). A surprising feature of TRPC4 and TRPC5 and heteromultimeric combinations of TRPC1 and TRPC5 is their strong potentiation (10–13) or activation (7) by micromolar concentrations of the trivalent lanthanoid cations La3+ and Gd3+, effects also seen for TRPC5 with millimolar Ca2+ (7, 13). Most other TRP channels, with the exception of TRPV1 (see below), and other Ca2+-permeable cation channels, including voltage-gated Ca2+ channels (14), are inhibited by micromolar concentrations of lanthanoids. In contrast to the potentiation of TRPC5 with micromolar concentrations of La3+ and Gd3+, millimolar concentrations cause inhibition (13). At the single channel level, the potentiatory and inhibitory effects of La3+ and Gd3+ overlap, with potentiation (an increase in channel open probability) dominating over inhibition (a decrease in conductance) at micromolar concentrations. With a mutational analysis, it was shown that two glutamate residues (Glu543 and Glu595; Fig. 1, A and B), at the start and close to the end of the pore-forming loop, are involved in potentiation. Neutralization of either of these amino acids led to a loss of potentiation, but inhibition persisted. A comparison of the sequences of other TRP channels with that of TRPC5 revealed the presence of glutamates at similar positions in TRPC4, consistent with the lanthanoid potentiation of this isoform (10, 11), but not in other TRPCs. Surprisingly, among the TRP channels, only TRPV1 has glutamates at analogous positions (Glu590 and Glu648; Fig. 1A), and
Glu\textsuperscript{648} is the first glutamate in an EFTE motif that is also present in TRPC4 and -5 (Fig. 1A). It is also particularly significant that Glu\textsuperscript{600} and Glu\textsuperscript{648} mediate proton (H\textsuperscript{+}) potentiation of heat- and capsaicin-activated currents and H\textsuperscript{+} activation of currents through TRPV1, respectively (15). A recent study demonstrated that TRPV1 is also lanthanoid (La\textsuperscript{3+} and Gd\textsuperscript{3+})-sensitive, an effect mediated by the same key residues that confer responsiveness to H\textsuperscript{+} (16). The presence of similar amino acids in analogous positions in the pore loops of TRPV1, TRPC4, and TRPC5 and the potentiatory effects of cations raise the question of whether TRPC5 can respond to H\textsuperscript{+}.

In this study, we therefore looked at the effects of H\textsuperscript{+} on TRPC5. We showed that currents through TRPC5 are potentiated by the extracellular H\textsuperscript{+} concentration in a range around the physiological pH. Using outside-out patch clamp recordings, it was shown that pH reductions lead to single channel currents through TRPC5 with longer open times and smaller amplitudes, an effect very similar to that of micromolar La\textsuperscript{3+} or Gd\textsuperscript{3+}. Using point mutants, we investigated the molecular determinants of potentiation of TRPC5 by H\textsuperscript{+}.

**EXPERIMENTAL PROCEDURES**

**Molecular Biology**—The cDNAs of TRPC5 and its pore mutants E543Q and E595Q/E598Q C-terminally fused to YFP were already used for previous studies from our laboratory. The isolation of the mouse TRPC5 coding sequence from brain total RNA (10), the introduction of the point mutations E543Q and E595Q/E598Q (13) and fusion of these cDNAs with the YFP gene (17) are described in the respective publications. The YFP-tagged versions were used in the experiments summarized in Figs. 2A, 3, and 4.

TRPC5wt-piRES2-EGFP, which was used in all other measurements on wild type TRPC5 (TRPC5-wt), was obtained by ligation of the 3-kb fragment from Spel/SacII restriction of TRPC5-wt-pcDNA3.1/V5-His-TOPO with NheI/SacII-linearized piRES2-EGFP (BD Biosciences Clontech). TRPC5 currents obtained from measurements using YFP-tagged TRPC5 (pcDNA3) were identical to those using the piRES2-EGFP vector system. Subcloning the NheI/Esp3I fragment of TRPC5-E543Q-pcDNA3 into TRPC5-wt-piRES2-EGFP led to TRPC5-E543Q-piRES2-EGFP, and the point mutations E595Q and E598Q and the double mutation E543Q/E595Q in TRPC5-piRES2-EGFP were introduced using the QuikChange site-directed mutagenesis kit (Stratagene, Amsterdam, The Netherlands) and appropriate primer sets. Sequences of the mutants were confirmed by DNA sequencing.

For experiments with TRPC4, we used the human β-splice variant fused C-terminally to YFP (11). Human TRPC6-piRES2-EGFP was a gift from V. Chubanov and contained the pIRES2-EGFP (BD Biosciences Clontech). TRPC6 cur-

**Cell Culture and Transient Transfection**—Human embryonic kidney cells (HEK 293) were maintained as described in the supplier’s recommendations. Transient transfections were performed in 35-mm dishes (Nunc, Wiesbaden, Germany) 1–2 days after plating using the FuGene6 (Roche Applied Science) or the TransIT (Mirus, Madison, WI) transfection kit. The transfected cells were trypsinized and seeded onto glass cover slips or directly into 35-mm Nunc dishes 2 days after transfection. The functional expression of the transfected cDNAs in HEK 293 cells was similar on both surfaces.

**Patch Clamp Recordings**—An EPC-7, EPC-9, or EPC-10 amplifier and Pulse software (HEKA, Lamprecht, Germany) were used for patch clamp recordings. For measurements performed in the whole cell configuration, patch pipettes, made from borosilicate glass (Science Products, Hofheim, Germany), had resistances of 3–6 megaohms when filled with the standard intracellular solution. Cells were clamped at a potential of −50 or −60 mV, as indicated in the figure legend of each experimental series. Current-voltage (I-V) relationships were obtained from voltage ramps from −100 to +100 mV with a duration of 400 ms applied every 4–7 s. Ramp data were acquired with a sampling rate of 5 kHz (EPC-7) or 20 kHz (EPC-9 and EPC-10) after filtering at 1 kHz (EPC-7) or 2.8 kHz (EPC-9 and EPC-10). The holding current was acquired at 30 Hz (X-Chart; HEKA). To quantify current potentiation and current inhibition observed by bath application of H\textsuperscript{+}, we interpolated currents before and after their application and normalized the values obtained in the presence of H\textsuperscript{+} to the interpolated values. Interpolation was done to avoid errors arising from the fact that currents through TRPC4, -5, and -6 decay with time. To determine pIC\textsubscript{50} and pEC\textsubscript{50} values, the concentration-response curves for H\textsuperscript{+} inhibition of TRPC6 (Fig. 2E) and TRPC5-E543Q (Fig. 3C) as well as for H\textsuperscript{+} activation of TRPC5-wt, -E543Q, and -E595Q/E598Q (Fig. 5D) were fitted with the Hill equation using the built-in fit routine in IGOR Pro 4.09 (Wavemetrics, Lake Oswego, OR).

All single channel recordings were performed in the outside-out configuration at −60 mV. Borosilicate glass pipettes had resistances between 5 and 8 megaohms when filled with the standard intracellular solution. Currents were acquired with a sampling frequency of 20 kHz after filtering at 4 kHz and stored discontinuously as sweeps with durations between 30 s and 2 min. For offline analysis, the single channel traces were filtered at 1 kHz. Events were detected by half-amplitude threshold analysis using a routine programmed in IGOR Pro 4.09. Idealization was subsequently tested by eye for a proper correlation with the acquired data. Single channel amplitudes were obtained by Gaussian fitting the all points histograms. Channel activity was expressed as nP\textsubscript{o}, the product of the number (n) of channels in the patch (obtained from the observed number of open levels) and the open probability (P\textsubscript{o}). nP\textsubscript{o} values were calculated for consecutive 0.5-s periods. Openings with durations shorter than 0.5 ms were excluded from the analysis. Because of extensive overlap of individual unitary current responses after application of H\textsuperscript{+} or Gd\textsuperscript{3+}, we used an algorithm established by Fenwick et al. (19) to obtain a reliable estimate of mean open times in the absence and presence of the effectors. The general applicability of the algorithm has been confirmed (20). The overall estimate of mean channel open time (t\textsubscript{j}) was calculated according to t\textsubscript{j} = (Σt\textsubscript{j})/N, where N is the number of all channel openings (transitions between a given level j and a subsequent level j + 1), t\textsubscript{j} designates the dwell time of a given level j, and the sum extends over all levels encountered in the recording. Values for t\textsubscript{j} were extracted from idealized traces generated in IGOR.
The standard extracellular solution contained 140 mM NaCl, 5 mM CsCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose, and 10 mM HEPES (pH 7.4 with NaOH). Extracellular solutions with pH values different from pH 7.4 had similar ionic compositions to the standard extracellular solution and were also titrated with NaOH to the final pH value but buffered with citrate (pH 4.2), MES (pH 5.5, 6.0, 6.3, 6.5 and 6.8), or HEPES (pH 7.0 and 8.0). Total Ca²⁺ and Mg²⁺ concentrations in citrate-buffered solutions were calculated using MaxChelator (Chris Patton, Stanford University, CA). The standard intracellular solution contained 110 mM cesium methane sulfonate, 25 mM CsCl, 2 mM MgCl₂, 3.62 mM CaCl₂, 10 mM EGTA, and 30 mM HEPES (pH 7.2 with CsOH) with a calculated free [Ca²⁺] of 100 nM. For the measurements summarized in Figs. 2 and 3, the pipette contained 1 mM instead of 10 mM EGTA and 0.362 mM instead of 3.62 mM CaCl₂. For AlF₄⁻ infusion, 2 μL of 0.5 mM NaF were mixed with 1 μL of 3 mM AlCl₃ and diluted with 100 μL of the pipette solution. The osmolarity of all solutions was between 290 and 310 mosmol/liter. All experiments were performed at room temperature (20–25 °C).

**Chemicals**—Gadolinium chloride and GTPγS were obtained from Sigma. All stock solutions were made in water and diluted to final concentrations in the bath or pipette solutions.

**RESULTS**

**Sequence Alignment of the Pore Regions of TRPC4, -5, and -6 and TRPV1**—A comparison of the amino acid sequences between their S5 and S6 segments reveals that TRPV1 and TRPC5 have negatively charged amino acids at only three analogous positions (Fig. 1A). Of these glutamates, one is located very close to S5, whereas the other two form part of an EFTE motif in the short stretch of amino acids between the putative pore and S6 (Fig. 1B). The functional relevance of the first and second of these glutamate residues for the channel-gating properties in the presence of La³⁺ and Gd³⁺ has previously been reported for both TRPV1 (Glu⁶⁴⁸ and Glu⁵⁴⁵) and TRPC5 (Glu⁵⁴³, Glu⁵⁴⁵, and Glu⁵⁹⁵) (13) and in the presence of H⁺ for TRPV1 (15). The proximal glutamate (Glu⁵⁴⁵) as well as the EFTE motif (starting with Glu⁵⁹⁴) are also present in TRPC4, consistent with the potentiation of this channel isoform by lanthanoids (10, 11). However, TRPC6, which is only inhibited by La³⁺ and Gd³⁺, lacks glutamate residues at positions similar to Glu⁵⁴⁵ and Glu⁵⁹⁵ in TRPC5.

**H⁺ Potentiation of G Protein-activated Currents through TRPC5**—We first tested G protein-activated TRPC5 currents for their sensitivity to the extracellular pH value. In whole cell patch clamp recordings from HEK 293 cells transiently transfected with TRPC5, the infusion of 500 μM GTPγS or 30 μM AlF₄⁻ into the cytoplasm via the patch pipette activated the channels and led to transient inward currents at negative holding potentials (with mean amplitudes of ~50.6 ± 6.9 pA/pF, n = 8, at −60 mV for GTPγS and −15.3 ± 3.2 pA/pF, n = 15, at −50 mV for AlF₄⁻). The I-V relationships of all of these currents were characteristic for some members of the TRPC channel family (e.g. see Ref. 21) and were doubly rectifying with a reversal potential close to 0 mV (Fig. 2A).

Decreasing the extracellular pH from 7.4 to 6.5 during the decaying phase of currents through TRPC5 led to significant current increases (Fig. 2A). Responses occurred within a few seconds of changing the extracellular pH and were reversible on returning to the standard extracellular solution (pH 7.4). It is notable that the effect was much more pronounced at negative
In further experiments to study the concentration dependence of these effects, we also tested intermediate pH values between pH 8.0 and 4.2. These data are summarized in Fig. 2D. Like pH 6.5, decreases in the extracellular pH from 7.4 to 7.0 or 6.0 led to a potentiation of the TRPC5-mediated currents that was much more pronounced at negative potentials. The largest potentiation was observed between pH 6.5 and 6.0. Under more acidic conditions, the potentiation of TRPC5 became weaker, and at pH 4.2, currents were inhibited in all experiments. Raising the pH from 7.4 to 8.0 significantly (p < 0.01) decreased TRPC5-mediated currents in both inward (to 56.0 ± 9.6%, n = 5, at −60 mV) (Fig. 2, A and D) and outward directions (to 76.6 ± 5.0% at +100 mV, n = 5).

These data show that the amplitude of inward currents through TRPC5 activated by G protein stimulation strongly depends on the extracellular pH value. The effects of H⁺ on TRPC5 are concentration-dependent and, like the effects of La³⁺ and Gd³⁺ on TRPC5 (13), have a biphasic characteristic, with currents increasing from alkaline pH values (pH 8.0) to maximum values between pH 6.5 and 6.0 and then decreasing with further reductions in pH to a nearly complete block at pH 4.2.

It has to be noted that two different kinds of endogenous currents were evoked by decreasing the extracellular pH value in recordings from both transfected and untransfected HEK 293 cells. A change to pH 6.5 or more acidic pH values was followed by very brief currents in the range of −0.2 to −1 nA at the holding potential (e.g. −14.0 ± 3.7 pA/pF with a duration of 2.2 ± 0.4 s at pH 5.5 in five of five control experiments at −50 mV). From their pH dependence, kinetics, and positive reversal potential, these currents clearly displayed all characteristics previously described for the endogenous acid-sensing ion channels (ASICs) in HEK 293 cells (22) and were largely prevented by the application of 30 μM amiloride. However, due to their rapid activation and inactivation, they only overlapped with the initial phase of the observed TRPC current potentiation if it all and thus were not routinely blocked. A second type of current, with strong outward rectification and a reversal potential close to 0 mV, was only observed at pH 4.2 and not at higher pH values (four of five control measurements on untransfected HEK 293 cells). This endogenous current was present for the whole time of H⁺ application and led, despite its relatively small inward component, to a slight underestimate of TRPC current inhibition in all pH-response curves at pH 4.2.

Effects of Extracellular H⁺ on Currents Mediated by TRPC4β—From similarities in their cDNA sequences, TRPC4 and TRPC5 are closely related channel subunits. This prompted us to compare the effects of H⁺ on currents through TRPC4 with those obtained for TRPC5. In whole cell patch clamp recordings from HEK 293 cells transiently transfected with TRPC4β, the intracellular infusion of 500 μM GTPγS led to transient inward currents with average current densities of −45 ± 13 pA/pF (n = 13) at a holding potential of −60 mV. As shown in Fig. 2B, the biophysical characteristics of TRPC4β were consistent with previous reports (10, 11). Like for TRPC5, currents through TRPC4β were potentiated by a reduction in extracellular pH from pH 7.4 to pH 6.5. At −60 mV, the currents were, on average, increased about 2-fold (to 188 ± 18%, n = 8, p < 0.001),
whereas the potentiation was less pronounced at positive potentials (to 115 ± 8% at +100 mV, n = 8, p < 0.01). The pH dependence of H⁺ potentiation of the TRPC4-mediated currents, shown in Fig. 2D, is very similar to that of TRPC5. However, compared with TRPC5, the acid potentiation of TRPC4β at pH 6.0 was much less, and at pH 5.5 currents were already inhibited. Thus, like for TRPC5, H⁺ dually affects TRPC4, causing current potentiation followed by current inhibition with decreasing pH values.

H⁺ Inhibition of TRPC6-mediated Currents—TRPC6 is more closely related to TRPC3 and -7 than to TRPC4 and TRPC5 and lacks glutamates at analogous positions to TRPC4 and -5 (Fig. 1A). We therefore compared the effects of low pH on TRPC6-mediated currents with those seen on TRPC5 (Fig. 2, C and E). Like for TRPC5, transient inward currents through TRPC6 were elicited by the infusion of AlF₄⁻ (~86.0 ± 2.4 nM, pH 7.4). In contrast to the effects of H⁺ on TRPC5-mediated currents, pH decreases only inhibited TRPC6, an effect that was already visible at pH 6.5 and increased with further reductions in pH. Both inward and outward currents were inhibited by reductions in pH. For TRPC6-mediated currents, half-maximal current inhibition at −50 mV was observed at pH 5.7 ± 0.1. These data indicate that the acid potentiation may be a specific effect limited to only a few members of the TRPC channel family.

Molecular Determinants of the Acid Potentiation of G Protein-activated Currents through TRPC5—As shown above, glutamate residues responsible for the lanthanoid potentiation of TRPC5 (Glu⁵⁴³ and Glu⁵⁹⁵) are located at similar positions in TRPC4 but not in TRPC6 (Fig. 1A), consistent with the presence and absence of this effect in the two isoforms, respectively (10, 11, 13). It was also shown (see above) that there is striking similarity between the H⁺ and lanthanoid effects on TRPC4, TRPC5, and TRPC6, indicating that the glutamate residues Glu⁵⁴³ and Glu⁵⁹⁵ may also play a role in the H⁺ sensitivity of TRPC5. The point mutants TRPC5-E543Q and TRPC5-E595Q were therefore studied for their responses to changes in extracellular pH. Like the wild type channel, both mutants could be activated by the infusion of 500 µM GTPγS in transiently transfected HEK 293 cells and showed current characteristics similar to those of the wild type (Fig. 3, A and B). It is notable that GTPγS-activated currents through TRPC5-E543Q (−110.7 ± 21.8 pA/pF, −60 mV, n = 7, p < 0.05 compared with TRPC5-wt) were significantly larger than those through TRPC5-wt (−50.6 ± 6.9 pA/pF, −60 mV, n = 8), whereas those through TRPC5-E595Q were significantly smaller (−22.1 ± 6.8 pA/pF, −60 mV, n = 21, p < 0.05 compared with TRPC5-wt; see inset to Fig. 3C).

Unlike TRPC5-wt (potentiation to 217 ± 24%, n = 8, at −60 mV), the currents through the proximal pore mutant E543Q were not potentiated by reductions in pH. In contrast, inhibition was already visible at pH 6.5 (to 83 ± 7%, n = 4, p < 0.05) and increased with further reductions in pH. Half-maximal inhibition by pH for this mutant was around pH 4.5. In contrast, with TRPC5-E595Q, a mutant of the glutamate residue distal to the putative pore loop, no significant changes in the responses of TRPC5-mediated currents to acid were observed. Like the wild type, the GTPγS-activated currents through this mutant increased when the extracellular pH value was reduced to pH values between 7.0 and 5.5 (Fig. 3C), and the largest potentiation was observed at pH 6.5 (199 ± 21%, n = 7, p < 0.001 compared with pH 7.4). In experiments to examine the possibility of an action of H⁺ on the second glutamate residue in the EFTE motif distal to the pore loop (Glu⁵⁹⁸, Fig. 1A), no differences between the responses of TRPC5-E598Q and TRPC5-wt to pH 6.5 were observed (251 ± 30%, n = 4, p = 0.45 compared with TRPC5-wt; data not shown). Similarly, TRPC5-E595Q/E598Q (13) was potentiated to 252 ± 35% at pH 6.5 (n = 7, p = 0.27 compared with TRPC5-wt; data not shown). At pH 4.2, the wild type channel and both distal pore mutants were blocked. The data above show that, like for La³⁺ and Gd³⁺ (13), neutralization of the residue Glu⁵⁴³ influences the effect of H⁺ on currents through TRPC5. Surprisingly, unlike lanthanoid effects on TRPC5, potentiation by H⁺ was neither reduced nor abolished by neutralizing either one or both of the distal glutamate residues Glu⁵⁹⁵ and Glu⁵⁹⁸.

FIGURE 3. Effects pH on GTPγS-activated currents through the TRPC5 mutants E543Q, E595Q, and E543Q/E595Q. A, pH 6.5 did not potentiate but weakly inhibited currents through TRPC5-E543Q. Left, time course of whole currents at the holding potential (−60 mV) at −100 mV (inset); right, I-V relationships at pH 6.5 and 7.4. B, pH dependence of the relative amplitude (to pH 7.4, 100%) of currents through TRPC5-E543Q (light gray), TRPC5-E595Q (dark gray), TRPC5-E543Q/E595Q (open), and TRPC5-wt (black). The inset shows the average maximum GTPγS-activated current densities at −60 mV and pH 7.4 from HEK 293 cells transfected with TRPC5-wt (black), TRPC5-E543Q (light gray), TRPC5-E595Q (dark gray), TRPC5-E543Q/E595Q (open).
Amino Acids Involved in Potentiation of Spontaneous Currents through TRPC5—We also tested the effects of H+ on basal currents through the mutants TRPC5-E543Q/E595Q and -E543Q/E598Q. Interestingly, TRPC5-E543Q, which showed no current increase on reducing pH when activated by G protein stimulation (Fig. 3), was potentiated by H+. As shown in Fig. 5A, short-term break-in, basal activity of TRPC5-E543Q was higher (−14.0 ± 3.2 pA/pF, n = 7, p = 0.01, −50 mV) than for the wild type (−5.2 ± 0.9 pA/pF, n = 9, −50 mV) and was further augmented by decreases in pH down to 6.0 (Fig. 5C). For the wild-type, the largest effects were observed at pH 6.5, with an average current density of −22.3 ± 6.1 pA/pF (n = 5). In contrast, potentiation of spontaneous currents through the distal pore mutant TRPC5-E543Q/E598Q by acid was much weaker than TRPC5-wt. Its basal activity was −0.9 ± 0.2 pA/pF (n = 5, p < 0.01, −50 mV) and thus significantly lower than TRPC5-wt (Fig. 5B and C, inset). At pH 5.5, where the largest activation was observed, the mean current density was only −2.6 ± 0.5 pA/pF (n = 11). Like TRPC5-E543Q/E598Q, the single mutant E595Q only showed weak effects in response to changes in extracellular pH (−0.3 ± 0.2 pA/pF at pH 6.5, n = 6), indicating that neutralization of Glu595 and not Glu598 influences the modulation of TRPC5 by acid pH. The small acid-induced increases in spontaneous currents through TRPC5-E543Q/E598Q and TRPC5-E543Q cannot be accounted for by poor expression of the mutants. GTPyS-activated currents were 40% of TRPC5-wt, whereas the maximum current activated by low pH in the absence of G protein stimulation was <10% of that of the TRPC5-wt current. To allow a rough comparison of the pH-response curves of the wild type and mutant channels, the steady-state currents were normalized to the maximally activated currents for each channel (at pH 6.5 for TRPC5-wt, -E543Q, and -E543Q/E595Q and at pH 5.5 for TRPC5-E595Q/E598Q, as shown in Fig. 5C). Interestingly, the pEC50 values for activation by acid were significantly different (Fig. 5D), with the activity of TRPC5-E543 shifted to more basic (pEC50 = 7.35 ± 0.19) and the activity of TRPC5-E543Q/E595Q/E598Q to more acidic pH values (pEC50 = 6.42 ± 0.08) compared with TRPC5-wt (pEC50 = 6.87 ± 0.02). The shifts may partly explain the differences in basal activities seen for the TRPC5 isoforms (Fig. 5C, inset).

The spontaneous activity of the double mutant TRPC5-E543Q/E595Q at pH 7.4 was high (−32.9 ± 8.3 pA/pF, n = 19, at −60 mV). This mutant responded to reductions in pH to 6.5

We also tested the response of the double mutant TRPC5-E543Q/E595Q (Fig. 3C). GTPyS-activated currents had a mean current density of −64.4 ± 12.5 pA/pF, n = 7, at −60 mV. Surprisingly, TRPC5-E543Q/E595Q was potentiated at pH 6.5 to 162.5 ± 13.3% (n = 4), a value not significantly different from TRPC5-wt (p = 0.15). Although currents through the double mutant increased in response to H+, potentiation by Gd3+ was not restored, and currents were inhibited in the range between 10 μM and 5 mM (data not shown).

Effects of H+ on Spontaneous Currents through TRPC5—We next tested whether spontaneous currents through TRPC5 respond to extracellular H+ without an activator of the PLC pathway. Spontaneous currents are characteristic for the murine clone (10, 13, 23) and are dependent on Ca2+ acting via calmodulin and myosin light chain kinase (24). A pH reduction from 7.4 to 6.5 indeed led to robust inward currents between −50 and −800 pA (−27.3 ± 5.3 pA/pF, n = 21) at a holding potential of −50 mV in HEK 293 cells transfected with the channel cDNA. The I-V relationships (Fig. 4A) during pH reduction were typical for TRPC5, and no comparable currents were observed in control cells (see above), indicating that the observed acid-evoked currents are conducted by TRPC5. The potentiation of spontaneous currents through mouse TRPC5 by acid differed from that of G protein-activated currents in two respects. First, the ratio of the current amplitudes of the acid-evoked currents (pH 6.5) to the basal activity at pH 7.4 (549 ± 57%, n = 13, −50 mV) was significantly larger (p < 0.001) than the corresponding ratio of the AlF4−- or GTPyS-induced currents at pH 6.5−7.4 (e.g. for AlF4−, 207 ± 12%, n = 9, −50 mV; Fig. 2, A and D). Second, in the case of spontaneous currents, a large increase in the inward and the outward currents was observed, whereas potentiation of G protein-activated currents was much stronger at negative potentials. For example, at pH 6.5, spontaneous currents at +100 mV were increased to 242 ± 27% (n = 6), whereas AlF4−-activated currents at the same potential were increased to only 117 ± 3%, effects that were significantly different (p < 0.001). Similar characteristics were also observed at other pH values between pH 8.0 and 5.5, a range over which currents were increased in a pH-dependent manner. As shown in Fig. 4B, TRPC5 was still basally active at pH 8.0 but less than at pH 7.4. Decreases in extracellular pH to values between 7.0 and 5.5 led to robust inward currents with the strongest changes in channel activity being observed at pH 6.5. At more acidic pH values, TRPC5 currents were slightly but not significantly decreased compared with currents at pH 6.5. These data show clearly that murine TRPC5 already responds to small changes in extracellular [H+] without application of an activator of G proteins.

FIGURE 4. Effects of pH on spontaneous currents through TRPCs. A, left, reductions in pH from 8.0 to 7.4, 7.0, and 6.5 led to increases in currents through TRPC5 at −50 mV and at ±100 mV (inset). Right, I-V relationships at pH 8.0 and 6.5 from the same experiment. B, pH dependence of the TRPC5 current density (pA/pF). *, significance relative to pH 7.4.
with an increase in current (to 261 ± 35% of that at pH 7.4, n = 16) that was significantly less than the increase seen for TRPC5-wt (p < 0.001). Larger decreases in pH to 5.5 resulted in a reduction in current compared with pH 6.5.

Comparison of the Acid and Lanthanoid Effects on Spontaneous Single Channel Currents through TRPC5—We next investigated the mechanisms underlying the effects of extracellular H+ on TRPC5 in more detail at the single channel level. Outside-out patch clamp recordings were performed to compare the single channel properties of TRPC5 in the standard extracellular solution (pH 7.4) and in the presence of 100 μM Gd3+ with those obtained at lower pH (pH 6.5). Under the same conditions used for the whole cell recordings described above (Fig. 4), single channel events of −2.46 ± 0.16 pA (−60 mV, 41.0 ± 2.6 picosiemens, n = 7) were observed at an extracellular pH of 7.4 in TRPC5-expressing (Fig. 6A) but not in untransfected cells, consistent with spontaneous activity of TRPC5 at the single channel (23) and macroscopic levels (see above). The mean open time (t_o) of TRPC5 under these conditions was 12.6 ± 3.9 ms (n = 5; Fig. 7). The reduction in extracellular pH to 6.5 led to marked changes in the single channel characteristics of TRPC5 with a reduction in the single channel amplitudes to −1.63 ± 0.14 pA (n = 7, p < 0.01 compared with pH 7.4), accompanied by a large increase in t_o (from 12.6 ± 3.9 ms at pH 7.4 to 56.4 ± 6.6 ms at pH 6.5, n = 5, p < 0.001; Fig. 7), which contributed, together with an increase in the number of openings, to the

FIGURE 5. pH effects on spontaneous currents through TRPC5 mutants. A, TRPC5-E543Q strongly responds to decreases in extracellular pH. Left, whole cell current at −50 mV and ±100 mV (inset). Right, I-V relationships of TRPC5-E543Q at pH 8.0 (black) and pH 6.5 (gray). B, TRPC5-E595Q/E598Q shows a much lower sensitivity to changes in extracellular pH than E543Q and the wild type. Left, whole cell current at −50 mV and ±100 mV (inset). Right, I-V relationships of TRPC5-E595Q/E598Q at pH 8.0 (black) and pH 6.0 (gray). C, pH dependence of TRPC5-wt (black), TRPC5-E543Q (light gray), TRPC5-E543Q/E595Q (open), and TRPC5-E595Q/E598Q (dark gray) activation. Base line-subtracted currents are given as current densities for each pH value. Inset, basal current densities (pA/pF) of TRPC5-wt (black), TRPC5-E543Q (light gray), and TRPC5-E595Q/E598Q (maxima, pH 6.5) and TRPC5-E595Q/E598Q (maxima, pH 5.5).

FIGURE 6. Effects of on basal (spontaneous) single channel currents through TRPC5 and TRPC5 mutants. A, H+ increases the open times and decrease the amplitude of currents through TRPC5. Top, excerpts from current traces from an outside-out patch showing spontaneous TRPC5 activity at pH 7.4 and 6.5 (−60 mV). Closed and open current levels are indicated by dashed and dotted lines, respectively. Bottom, channel activity from longer periods of the same experiment expressed as nPo over time. Inset, all points histograms obtained from current traces at pH 7.4 and 6.5. B, Gd3+ increases the open time and decreases the amplitude of TRPC5. Single channel traces of TRPC5 activity in the control (pH 7.4, left) and in the presence of 100 μM Gd3+ (right) at −60 mV. C–E, examples of nPo plots (top graphs) and single channel activity (bottom traces) at pH 7.4 (left) and pH 6.5 (right) for the mutants TRPC5-E543Q, E595Q, and E543Q/E595Q.
dramatic increase in $nPo$ from $0.03 \pm 0.01$ at pH 7.4 to $0.69 \pm 0.18$ at pH 6.5, $n = 5$, $p < 0.01)$. Consistent with data published previously (13), similar changes were also observed when Gd$^{3+}$ (100 $\mu$M) was added to the standard extracellular solution (Figs. 6B and 7). Thus, the single channel data provide further evidence for similarities in the effects of H$^+$ and Gd$^{3+}$.

**Effects of pH on the Single Channel Activity of TRPC5 Mutants**—We also looked at the effect of lowering the pH on spontaneous activity of TRPC5-E543Q, -E543Q/E595Q, and -E595Q at the single channel level (Figs. 6 and 7).

In line with previous data on the mutant TRPC5-E595Q/ E598Q (13), the mutants in which E595 was neutralized, TRPC5-E595Q and TRPC5-E543Q/E595Q, had higher single channel current amplitudes at $-60$ mV and pH 7.4 (TRPC5-E595Q, $-3.41 \pm 0.05$ pA, $n = 5$; TRPC5-E543Q/E595Q, $-3.31 \pm 0.16$ pA, $n = 6$) than TRPC5-wt ($p < 0.01$ in all cases). As reported previously (13), TRPC5-E543Q had an amplitude similar to that of TRPC5-wt. The mutants TRPC5-E543Q and TRPC5-E543Q/E595Q had noticeably higher levels of spontaneous channel activity than TRPC5-wt (Figs. 6C and E, and 7). The increase in $nPo$ resulted from an increase in the number of openings rather than $t_o$ (Fig. 7). Consistent with the whole cell data, TRPC5-E595Q had very low levels of spontaneous activity at pH 7.4 (Fig. 6D), too low for a reliable analysis.

Upon decreasing the pH to 6.5, channel activity increased in some patches from cells expressing TRPC5-E543Q and TRPC5-E543Q/E595Q. The increases occurred because of an increase in the number of openings, but not in $t_o$. However, as the whole cell recordings with these mutants show (Fig. 5C), the increases in currents in response to this pH change are not as large as for TRPC5-wt. Because of this and the higher, more variable activity at pH 7.4, the increase in $nP_o$ was not statistically significant (Fig. 7). For TRPC5-E543Q, the decrease in pH reduced the single channel amplitude (by 26%, $p < 0.05$) and to a similar extent ($p = 0.47$) to TRPC5-wt (33%). In contrast, for mutants containing E595Q, the amplitude was not significantly reduced ($p = 0.4$ and 0.56 for E595Q and E543Q/E595Q, respectively). Thus, larger unitary currents, which do not decline as strongly upon lowering pH, will contribute to the ensemble current through E543Q/E595Q.

**DISCUSSION**

In this study, we show for the first time that the classical TRP channel TRPC5 can be gated by H$^+$. This adds an additional, potentially physiologically important modulator to the growing list of substances that can regulate this channel. It is generally accepted that TRPC5 is a receptor-operated channel activated intracellularly by signaling downstream of PLC (10, 27), either by an unidentified messenger or possibly by depletion of intracellular Ca$^{2+}$ stores. The latter mechanism has been shown for some species variants (7, 28) but was not seen in others (for reviews, see Refs. 6 and 9). Later studies also demonstrated activation by extracellular Gd$^{3+}$ and Ca$^{2+}$ (7), the physiological significance of which remains elusive. More recently, it was shown that TRPC5 can be activated by lysophospholipids (8) and by cysteine S-nitrosylation (29), mechanisms that may be physiologically significant.

Inward current amplitudes of both G protein-activated and basal TRPC5 currents were strongly dependent on the extracellular pH and increased from pH 8 to maximum values around pH 6.5. Further decreases in pH reduced the maximum amplitudes. These effects of H$^+$ show striking similarities to those of La$^{3+}$ and Gd$^{3+}$, which also activate and modulate currents through TRPC5 in a biphasic manner with potentiatory and inhibitory components at micro- and millimolar concentrations, respectively (7, 13). Further evidence for similar actions of Gd$^{3+}$ and H$^+$ was obtained in the present study at the single channel level. Both decreased the amplitude of single channel currents through TRPC5 while, at the same time, increasing the $t_o$, the latter, together with an increase in the number of channel openings, contributing to an increase in $nP_o$.

Mutations of glutamate residues that are essential for the positive modulation of TRPC5 by lanthanoids modified the effects of H$^+$ on TRPC5. However, the effects of H$^+$ are more complex. Our evidence points to a role of the proximal glutamate Glu$^{543}$ in the modulation of channel activity by H$^+$. Mutation of this amino acid to glutamine, mimicking neutralization by H$^+$, resulted in an increase in spontaneous channel activity and G protein-activated currents. pH reductions that potentiated TRPC5-wt were no longer able to potentiate currents under conditions of strong channel activation (E543Q plus G protein stimulation) but were still able to potentiate spontaneous currents. The potentiation of spontaneous currents remaining in TRPC5 mutants with E543Q indicates that H$^+$ must also act at least one other site. The reduction in potentiation and increased spontaneous activity in channels with this mutation indicate that neutralization of the amino acid by...
**H⁺ and TRPC5**

Mutation may result in partial potentiation, thus reducing the additional current that can be induced by H⁺. In contrast to the potentiatory effect of lanthanoids, which was also abolished in E595Q, potentiation of G protein-activated currents by H⁺ was largely unaffected by this mutation. This difference could be explained by the relaxed configuration and the large size of the lanthanoid ions that may require more than one negative residue for a stable interaction with the channel. Based on the crystal structure of distantly related K⁺ channels (e.g., see Ref. 30), it is indeed possible that the residues at opposite ends of the pore loop can come into close proximity at the extracellular membrane surface. In contrast to lanthanoids, H⁺ is very small, carries only a single charge, and is capable of neutralizing the target residue without auxiliary binding sites. We therefore suggest that the potentiatory component of TRPC5 current modulation by La³⁺, Gd³⁺, and Ca²⁺ and part of that by H⁺ is promoted by the neutralization of Glu⁵⁴³, whereas Glu⁵⁹⁵ may be required by the larger cations as a binding stabilizer. The mutation E595Q or E595Q/E598Q did not influence the effect of H⁺ on G protein-activated currents but did decrease potentiation of basal current activity. However, some potentiation of basal currents persisted. The presence of H⁺-induced potentiation of both G protein-activated and basal currents in the double mutant E543Q/E595Q is a clear indication that other, yet unidentified sites are also involved in current increases in response to H⁺. A preliminary screen of mutants (Glu to Gln or Asp to Asn) of all other acidic amino acids present in the pore region of TRPC5, including Glu⁵⁵⁹ and Glu⁵⁷⁰, which are conserved in TRPC4 (Glu⁵⁵⁸ and Glu⁵⁶⁹; Fig. 1A), failed to find marked changes in the response to lowering the pH to 6.5 (data not shown). The effects of the E543Q/E595Q mutant indicate that there may be an interaction between these amino acids in the regulation of TRPC5.

Further insight into the effects of H⁺ on TRPC5 were also gained by studying the effects of pH 6.5 on the spontaneous activity of the mutant channels at the single channel level. The spontaneous activity of the mutants was consistent with the observations in whole cell recordings, with E543Q and E543Q/E595Q showing higher activity and E595Q showing lower activity. The higher activity of mutants containing E543Q was not caused by an increase in open time, like that seen with H⁺ or lanthanoids on TRPC5-wt, but only by an increase in the number of openings. In those experiments, in which larger increases in **p**H were observed on reducing pH, there was no increase in **t**ₚ, comparable with that in TRPC5-wt.

The mutation E543Q mimics part of the response of TRPC5 to H⁺, by increasing spontaneous channel activity, but does not produce the increase in channel open time characteristic for the effects of lanthanides and H⁺ on TRPC5. Dramatic increases in channel open time like those seen with lanthanoids or H⁺ are not observed during TRPC5 activation (e.g., by application of GTPγS to inside-out patches) (10) or in outside-out patches after activation of TRPC5 by GTPγS, where **t**ₚ values are not significantly different from those of spontaneously active channels described here.³ In the latter, decreasing the pH to 6.5 had effects on channel kinetics similar to those seen for spontaneous channel activity.

The potentiatory effects of H⁺ on TRPC5 resemble those on TRPV1. For unitary currents through TRPV1, there is also an increase in the length of channel openings accompanied by a decrease in unitary current amplitude (31, 32). For TRPV1, which has glutamate residues involved in H⁺ action at analogous positions to TRPC5 (see Fig. 1A), neutralization of these amino acids has also been shown to site-specifically diminish or abrogate potentiation (TRPV1-E600Q) and activation (TRPV1-E648Q) by acid, suggesting that activation and potentiation of TRPV1 by low pH are distinct processes (15). However, another study found more complex effects, with both amino acids affecting activation by H⁺ (16). In addition, the latter study showed that Glu⁶⁴⁸ is involved in activation and potentiation of TRPV1 by Gd³⁺, whereas Glu⁶⁴⁸ as well as involvement in potentiation. Gd³⁺, like H⁺ (31, 32), also had inhibitory effects on TRPV1 (16). There are, therefore, several similarities between the effects of H⁺ and lanthanoids on TRPV1 and TRPC5 and the possibility of common mechanisms being involved. A change in gating kinetics is responsible for the potentiation and a reduction in unitary current for inhibition. For TRPCs, inhibition by trivalent cations or H⁺ is probably a feature of most isoforms, as demonstrated at the whole cell level for TRPC6 (this paper) (13).

Although activation and potentiation may be separable for TRPV1, for TRPC5 at the single channel level, potentiation of spontaneous currents by H⁺ and Gd³⁺ was qualitatively and quantitatively very similar to the lanthanoid potentiation of G protein-activated currents described previously (13).

The mechanism by which Ca²⁺, La³⁺, Gd³⁺, and H⁺ influence the activity of TRPC5 remains unclear. Binding of these cations to the Glu⁵⁴³ at the extracellular surface of the channel could, by neutralizing the negative charge, allosterically modulate the affinity of TRPC5 for its putative endogenous agonist, which most likely acts from the inside, leading to channel potentiation. This hypothesis has been proposed for the action of H⁺ on the effects of capsaicin on TRPV1 (15, 32). However, a study with TRPV1 antagonists found no evidence for a change in the conformation of the capsaicin binding pocket at low pH (33). Since TRPV1 is potential-dependent, and activators like heat or capsaicin shift the potential dependence of channel activation to more negative potentials (e.g., see Ref. 34), the effect of cations on TRPV1 could also be explained by negative shifts in the potential dependence of channel activation. This effect would have to result from a modification of gating rather than a change in surface potential, which would shift in the wrong direction. A potential dependence of channel activation would explain the effects of lanthanides and H⁺ on TRPC5. Although these TRP isoforms have not explicitly been shown to be potential-dependent, the change in shape of the I-V relationship of TRPC4 and TRPC5 during G protein-mediated current activation, with the inward current becoming more linear with stronger activation (9, 35), can readily be explained by a shift in potential-dependent channel activation to more negative potentials. Differences in the extent of channel activation at the time point of lanthanoid or H⁺ application could explain the potential dependence of the cation effects. If the channel is

³ M. Semtner and T. D. Plant, unpublished observations.
strongly activated (e.g. by G protein stimulation) (Fig. 2A) and the conductance at positive potentials is close to its maximum, inward currents will be affected more than outward currents, whereas, at low levels of activation (e.g. in the absence of G protein stimulation) (Fig. 4A), shifts in the potential dependence of activation will increase the conductance, and thereby the currents, at both negative and positive potentials. In this scenario, mutations that affect binding or the potential dependence of channel activation could influence the effect of lanthanides or H⁺. Evidence for an effect on TRPC5 in addition to a shift of potential dependence is the large increase in channel open time, which has not been observed during channel activation. Since, in addition to the residues identified to be involved in the effects of lanthanides and H⁺, cysteines located in this region of the channel can also modify channel activity (29), there is the possibility that complex interactions between amino acids in the pore loop are involved in the regulation of channel activity.

Although not investigated in such detail, TRPC4 is also likely to be modulated by a similar mechanism to TRPV1 and TRPC5, since it displays potentiation by H⁺, La⁺, and Gd³⁺ (this paper) (10, 11). Other TRP channels for which activation or potentiation by low pH have been described are TRPV4 and TRPM7, respectively. However, although currents through the former have been reported to be increased at pH values of <7 (36), we saw no effect of reducing the pH to 5.5 on human TRPV4 (37). H⁺ potentiates inward currents through TRPM7 by competing with divalent cations for binding sites probably located within the channel pore (37). Since these effects have also been observed for endogenous Mg²⁺-inhibited channels thought to be TRPM7, and these are present in HEK 293 cells (e.g. see Ref. 38), this channel may be responsible for the endogenous current seen at pH 4.2.

Most other cation-permeable channels, including voltage-dependent Na⁺, Ca²⁺, K⁺, glutamate receptor, and cyclic nucleotide-gated channels, are inhibited by H⁺. Exceptions are members of the ASIC family, which are cation channels opened by relief from inhibition by Ca²⁺ on H⁺ binding (39). However, since raising extracellular Ca²⁺ has potentiatory rather than inhibitory effects on TRPV1 and TRPC5 (13, 40), relief from divalent block is an unlikely mechanism for the stimulatory effects of H⁺ on these channels. ASIC1a is endogenously expressed in HEK 293 cells (22), and ASIC-mediated currents were visible in the present study upon decreases in pH to ≤6.5. Depending on the variant, homomeric ASICs differ in their sensitivity to pH. ASIC3 is the most sensitive to reductions in pH 7.4 and is activated at <7.1. Because of the steep pH dependence of ASIC activation, further small decreases lead to dramatic increases in current. Mouse TRPC5 is sensitive to pH changes around 7.4. Spontaneous channel activity is reduced upon raising the pH and increases upon lowering the pH. In this respect, it differs from TRPV1, which shows little spontaneous activity and is only activated at pH <6.5.

The response of TRPV1 to protons is of major importance for the sensitivity of nociceptive neurons to changes in tissue pH, and TRPV1 carries most of the pH-gated current in these neurons (41). In contrast, little is known about the physiological role of TRPC5. Studies looking at the expression pattern of TRPC mRNA revealed that TRPC5 is highly expressed in brain but is also detectable at lower levels in some peripheral tissues (e.g. see Refs. 27, 28, and 42). TRPC4 is more widely expressed (e.g. see Refs. 42 and 43). Currents in cells from native tissues that might be carried by TRPC4 and/or TRPC5, or heteromultimers of these channels with TRPC1 have been reported (e.g. for a review, see Ref. 6). Our results suggest that, in addition to regulation by G protein-coupled receptors and receptor tyrosine kinases, the activity of TRPC5 may be regulated by small variations in pH around physiological values and that stronger decreases in pH are capable of activating quite large currents. We hypothesize that TRPC5 could act as a pH sensor linking extracellular acidification to channel activation, cell depolarization, and Ca²⁺ entry, the latter through both the channel itself and, if present, through voltage-gated Ca²⁺ channels activated by the depolarization. In contrast to our suggestion, one study looking at the effects of H⁺ on muscarinic acetylcholine receptor-activated cationic currents in guinea pig ileal smooth muscle cells, which are most likely carried by TRPC4 (44), found that cationic currents were inhibited by lower pH values and potentiated in more alkaline extracellular milieu (45).

In conclusion, we show that the activity of TRPC5 is increased by decreasing the extracellular pH and can add H⁺ to the increasing number of regulators of TRPC5. Further studies are necessary to identify cells expressing TRPC5 in the brain or other tissues and investigate the role of changes in pH in the regulation of the activity of native channels with a contribution of TRPC4 or TRPC5.

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