Mechanism and Molecular Determinant for Regulation of Rabbit Transient Receptor Potential Type 5 (TRPV5) Channel by Extracellular pH

Byung-II Yeh, Tie-Jun Sun, Jason Z. Lee, Hsi-Hsien Chen, and Chou-Long Huang

The transient receptor potential type 5 (TRPV5) channel is present in kidney and intestine and important for transepithelial (re)absorption of calcium in these tissues. We report that in whole-cell patch clamp recording extracellular acidification inhibited rabbit TRPV5 with apparent $pK_a$ $\approx -6.55$. The two extracellular loops between the fifth and sixth transmembrane segments of TRPV5 presumably form part of the outer opening of the pore and likely are important in binding and regulation by external protons. We found that replacement of glutamate 522 to glutamine (E522Q) decreased the sensitivity of the channel to extracellular acidification. Mutations of other titratable amino acids within the two extracellular loops to non-titratable amino acids had no effect on pH sensitivity. Substitutions of aspartate or other titratable amino acids for glutamate 522 conferred an increase in pH sensitivity. The pH sensitivity mediated by glutamate 522 was independent of extracellular or intracellular Mg$^{2+}$. Single channel analysis revealed that extracellular acidification reduced single channel conductance as well as open probability of the wild type channel. In contrast to wild type channel, extracellular acidification did not reduce open probability for E522Q mutant. Methanethiosulfonate reagents inhibited the activity of glutamate 522 to cysteine mutant channel with a reaction rate constant approaching that with free glutamate. The amount of Ca$^{2+}$ excreted by the kidney is about 2% of the total filtered load (3). About 98% of the filtered Ca$^{2+}$ is reabsorbed by the kidney. The absorption of Ca$^{2+}$ in intestine and the reabsorption in kidney occur via both paracellular and transcellular pathways. In the kidney, the transcellular reabsorption of Ca$^{2+}$ occurs mainly in the distal convoluted tubule (DCT) and accounts for $\sim 15$–$20\%$ of total reabsorption along the tubule. The relative contribution of transcellular versus paracellular absorption of Ca$^{2+}$ along the intestinal tract is less clear.

The transcellular (re)absorption of Ca$^{2+}$ is a multistep process (3). It begins with passive entry of Ca$^{2+}$ through the Ca$^{2+}$ channels in the apical membranes followed by diffusion of Ca$^{2+}$ through cytosol facilitated by binding to Ca$^{2+}$–binding protein calbindin-D$_{28K}$ and eventually extrusion of Ca$^{2+}$ across the opposing basolateral membranes. The extrusion of Ca$^{2+}$ across the basolateral membranes requires energy and is mediated by Na$^+$/Ca$^{2+}$ exchangers and Ca$^{2+}$-ATPases operating against the electrochemical gradient for Ca$^{2+}$. It is believed that the initial step of passive entry through Ca$^{2+}$ channels in the apical membranes is likely the rate-limiting step of the transepithelial Ca$^{2+}$ reabsorption in the distal nephron (3).

Several cDNAs for apical Ca$^{2+}$ channels have been recently isolated from epithelial tissues. Hoenderop et al. (4) isolated a cDNA from rabbit kidney and named it ECaC1 (for epithelial Ca$^{2+}$ channel). Northern blot analysis revealed that ECaC1 message is expressed in kidney, small intestine, and placenta. In the kidney, ECaC1 is localized to the apical membranes of DCT by immunofluorescent straining (4, 5). Peng et al. (6, 7) isolated CaT1 (for Ca$^{2+}$ transporter protein) and CaT2 from rat intestine and kidney, respectively. CaT1 is also known as ECaC2. CaT2 is the rat ortholog of ECaC1. The ECaC/CaT channels belong to the superfamily of cation-permeable ion channels known as transient receptor potential (TRP) (8). The TRP superfamily of ion channels can be divided into several families. The TRPV family is named after its first member, capsaicin (vanilloid) receptor (9). ECaC1/CaT2 and ECaC2/CaT1 are now known as TRPV5 and TRPV6, respectively (8).

Calcium (Ca$^{2+}$) is the most abundant cation in the human body and critical for many processes such as bone mineralization, formation of blood clots, regulation of cell-cell adhesion, and intracellular signaling (1, 2). Although the majority of Ca$^{2+}$ is present in the bone and is in continuous turnover, there is little or no net gain or loss of Ca$^{2+}$ from bone in normal young and healthy adults. To maintain calcium balance, the kidney excretes the same amount of calcium absorbed by the intestine. The amount of Ca$^{2+}$ excreted by the kidney is about 2% of the total filtered load (3). About 98% of the filtered Ca$^{2+}$ is reabsorbed by the tubule. The absorption of Ca$^{2+}$ in intestine and the reabsorption in kidney occur via both paracellular and transepithelial pathways. In the kidney, the transcellular reabsorption of Ca$^{2+}$ occurs mainly in the distal convoluted tubule (DCT) and accounts for $\sim 15$–$20\%$ of total reabsorption along the tubule. The relative contribution of transepithelial versus paracellular absorption of Ca$^{2+}$ along the intestinal tract is less clear.

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The abbreviations used are: DCT, distal convoluted tubule; TRP, transient receptor potential; TRPV, V type subfamily of TRP channels; CHO, Chinese hamster ovary; ECaC, epithelial Ca$^{2+}$ channel; CaT, Ca$^{2+}$ transporter protein; MTS, methanethiosulfonate; pH, extracellular pH; BAPTA, 1,2-bis(2-aminoethoxy)ethane-N,N,N',N'-tetraacetic acid; MTS-SEA, methanethiosulfonate ethylammonium; MTSET, methanethiosulfonate ethyltrimethylammonium; MTSES, methanethiosulfonate ethylsulfonate; I-V, current-voltage.
Overall cDNAs for TRP channels encode polypeptides of −700–1,000 amino acids with amino acid homology. Hydrophobicity analysis of the TRPV5 and TRPV6 polypeptides predicts a transmembrane topology of an amino-terminal cytoplasmic region containing many ankyrin repeats, six membrane-spanning domains with a putative pore-forming region similar to other Ca2+-permeable channels, and a carboxyl-terminal cytoplasmic entrance of the pore and causes Ca2+-dependent inactivation (20, 21). We used Na+ ions as charge carriers for inward currents to distinguish the direct effect of pH, on TRPV5 activity from other potential effects through alteration of Ca2+-dependent inactivation. Current-voltage (I-V) relationships were recorded using a voltage ramp protocol (−100 to 100 mV over 400 ms applied every 15 s from 0-mV holding potential) (Fig. 1B). Currents through TRPV5 were strongly inwardly rectifying and reversed at −15 mV at pH 7.4 (Fig. 1C, see Fig. 6 for better illustration of reversal potential). Substitution of NaCl for NaAsp in bath solution did not alter the I-V relationship or reversal potential significantly (not shown). The effects of pH, on TRPV5 were examined by titrating HEPES in bath solution to different pH values. As shown in Fig. 1, C and D, decreasing pH, stepwise from 8.4 (maximal current) to 4.4 caused a progressive reduction in currents. The acid-induced inhibition was reversible; currents recovered as pH, was increased (Fig. 1D). The apparent pH, for acid inhibition of the current was estimated at 6.55 ± 0.05 (Fig. 1C). This value of apparent pH, is within the range of pH (6−7) in the luminal fluid of DCT in physiological and pathophysiological states (22).

Identification of Amino Acid of TRPV5 Involved in Mediating Inhibition of the Channel by Extracellular Acidification—The predicted transmembrane topology of TRPV5 resembles voltage-gated cation channels and contains six transmembrane-spanning domains and a putative pore region between the fifth and sixth domains (11, 12). The two extracellular loops between the putative pore region and the last two transmembrane domains presumably form part of the outer vestibule of the pore (Fig. 2A). Proton-titratable amino acids in these loops are likely candidates for sensing and mediating the regulation of channel function by protons. To identify the amino acids responsible for proton sensing, we mutated each of the eight titratable amino acids in the two extracellular loops to a closely related non-titratable residue (Fig. 2A) and examined the sensitivity of each of the mutants to inhibition by extracellular acidification (Fig. 2B–H). We found that mutation of glutamate 522 to glutamine (E522Q) decreased the sensitivity of the channel to inhibition by extracellular acidification (Fig. 2E). The apparent pH, for acid inhibition was shifted toward acidic pH by 1.2 pH units (pH, 6.55 ± 0.05 for WT versus 5.32 ± 0.1 for E522Q; p < 0.01). These results suggest that titration of glutamate 522 by protons is involved in pH, regulation of TRPV5. The pH, of free glutamic acid is ~4 (23). The difference between the pK, of TRPV5 conferred by glutamate 522 and that of a free glutamate in solution is likely due to the fact that titratable groups

**EXPERIMENTAL PROCEDURES**

**Molecular Biology—Nucleotide coding sequences of cDNAs for rabbit (4) and human TRPV5 (14) were obtained by polymerase chain reaction using rabbit and human kidney cDNA as templates, confirmed by nucleotide sequencing, and inserted into pCDNA3 mammalian expression vector for transient expression in Chinese hamster ovary (CHO) cells. Site-directed mutagenesis of TRPV5 was performed using a commercial mutagenesis kit (QuikChange from Stratagene, La Jolla, CA) and confirmed by sequencing (15, 16).

**Cell Culture and Patch Clamp Recording in Cultured Cells—**CHO-K1 clone (from ATCC) was cultured in F12-K medium (Invitrogen) containing 10% fetal calf serum. Cells (at −50% confluence) were co-transfected with cDNA for pEGFP plus cDNAs for wild type or mutant TRPV5 using LipofectAMINE Plus transfection kits (Invitrogen) and the protocol provided by the manufacturer's instruction manual. About 24 h after transfection, cells were dissociated by limited trypsin treatment and placed in a chamber for recording. Transfected cells were identified by epifluorescence microscopy.

Whole-cell currents were recorded with an Axopatch 200B patch clamp amplifier (Axon Instruments) as described previously (17). Pipette solution contained 1 mM MgCl2, 4 mM NaAsp, 10 mM BAPTA, 130 mM CsAsp (cesium aspartate), 10 mM CsCl, 10 mM HEPES (pH 7.4). Bath solution contained 140 mM NaAsp, 10 mM NaCl, 1 mM EDTA, and 10 mM HEPES at different pH values as specified. For experiments in Fig. 6, pipette solution contained 130 mM CsAsp, 10 mM CsCl, 10 mM EDTA, and 10 mM HEPES (pH 7.2). Bath solution contained 140 mM NaAsp, 10 mM NaCl, 1 mM EDTA, and 10 mM HEPES at different pH values as specified. The voltage protocol used for each experiment is described in the individual figure. Pipette tip resistance ranged from 5 to 10 megohms. Capacitance and access resistance were monitored. Stock solutions for MTS reagents (Toronto Research Chemicals, North York, Ontario, Canada) were dissolved in bath solution prior to each experiment. For cell-attached single channel recording (18), pipette and bath solution contained potential regulatory sites for protein kinases (10, 11).

**Data Analysis and Statistics—**To calculate the apparent second-order rate constant for inhibition of channels by MTS reagents, the time constant was obtained by fitting the time course of inhibition of channels by MTS reagents with a single exponential. The rate constant was calculated by dividing the reciprocal of the time constant by the concentration of reagent (19). To analyze the sensitivity of the channel to inhibition by extracellular protons, relative currents at different pH, values were fitted with a modified Hill equation using the Sigma-Plot program (18). Data are shown as mean ± S.E. of a number of observations. Statistical comparison was made using unpaired Student’s t test.

**RESULTS**

**Inhibition of TRPV5 by Extracellular Acidification**—The activity of rabbit TRPV5 expressed in CHO cells was measured using ruptured whole-cell recording (Fig. 1A). Similar to other Ca2+-channels, TRPV5 conducts monovalent cations if Ca2+ ions are removed from solution (12). Also Ca2+ entry through TRPV5 raises intracellular Ca2+ in a microdomain near the cytoplasmic entrance of the pore and causes Ca2+-dependent inactivation (20, 21). We used Na+ ions as charge carriers for inward currents to distinguish the direct effect of pH, on TRPV5 activity from other potential effects through alteration of Ca2+-dependent inactivation. Current-voltage (I-V) relationships were recorded using a voltage ramp protocol (−100 to 100 mV over 400 ms applied every 15 s from 0-mV holding potential) (Fig. 1B). Currents through TRPV5 were strongly inwardly rectifying and reversed at −15 mV at pH 7.4 (Fig. 1C, see Fig. 6 for better illustration of reversal potential). Substitution of NaCl for NaAsp in bath solution did not alter the I-V relationship or reversal potential significantly (not shown). The effects of pH, on TRPV5 were examined by titrating HEPES in bath solution to different pH values. As shown in Fig. 1, C and D, decreasing pH, stepwise from 8.4 (maximal current) to 4.4 caused a progressive reduction in currents. The acid-induced inhibition was reversible; currents recovered as pH, was increased (Fig. 1D). The apparent pH, for acid inhibition of the current was estimated at 6.55 ± 0.05 (Fig. 1C). This value of apparent pH, is within the range of pH (6−7) in the luminal fluid of DCT in physiological and pathophysiological states (22).
of amino acids within polypeptide chains are influenced by the local chemical environment (24, 25). Mutations of other titratable amino acids did not alter pH sensitivity of the channels (Fig. 2, B–D and F–H). Currents of tyrosine 547 to phenylalanine mutant (Y547F) in CHO cells were unstable, and thus the effects on this mutant were not studied (not shown).

Inhibition of TRPV5-mediated $^{45}$Ca$^{2+}$ Uptake by Extracellular Acidification—The physiological role of TRPV5 is to conduct Ca$^{2+}$ (11, 12). We examined the effects of pH on Ca$^{2+}$ permeation through TRPV5. We studied Ca$^{2+}$ permeation through the channel using $^{45}$Ca$^{2+}$ radioactive isotope uptake. $^{45}$Ca$^{2+}$ uptake in Xenopus oocytes was used for expression cloning of cDNAs for ECaC/CaT (4, 6). We chose the Xenopus oocyte expression system for $^{45}$Ca$^{2+}$ uptake studies because of its reliability and high signal/background ratio. Oocytes expressing TRPV5 were preincubated in nominal Ca$^{2+}$- and Mg$^{2+}$-free solution for –6 h and incubated with $^{45}$Ca$^{2+}$ (1 μCi/ml) for measurement of uptake over time as indicated (Fig. 3A). Oocytes expressing TRPV5 exhibited time-dependent uptake over 1 h (Fig. 3A). Control H$2$O-injected oocytes did not show significant uptake above background (not shown). TRPV5-mediated $^{45}$Ca$^{2+}$ was more than 90% inhibited by La$^{3+}$ (Fig. 3A). Similar to the results of whole-cell recording studies, extracellular acidification from pH 9 to 4 decreased TRPV5-mediated $^{45}$Ca$^{2+}$ uptake (Fig. 3B). Mutation of glutamate 522 shifted the pH sensitivity of TRPV5-mediated $^{45}$Ca$^{2+}$ uptake toward acidic pH (apparent p$K_a$ 6.21 ± 0.25 for WT versus 5.12 ± 0.18 for E522Q; p < 0.05).

Effects of Substitution of Other Titratable Amino Acids for Glutamate 522 on pH Sensitivity—To further evaluate the importance of titration of glutamate 522 for inhibition of TRPV5 by pH$e$, we replaced glutamate 522 by several amino acids with a different p$K_a$ value and ionic charge in the titratable group. The p$K_a$ and charge of titratable groups of these amino acids are shown in Fig. 4A. Glutamate and aspartate have the same titratable group, carboxyl. Accordingly glutamate 522 to aspartate mutant (E522D) had virtually the same pH$e$ sensitivity as the wild type TRPV5 (Fig. 4, B and C). Amino acids histidine, cysteine, tyrosine, and lysine are also titratable by protons. We found that the apparent p$K_a$ values for pH$e$ regulation of E522H, E522C, and E522Y mutants were 6.2 ± 0.15, 6.69 ± 0.01, and 7.30 ± 0.01, respectively (Fig. 4, B and C). These p$K_a$ values were all significantly more alkaline than that for E522Q mutant (p < 0.05 versus p$K_a$ 5.32 ± 0.1 for E522Q), which contains the non-titratable glutamine. Cells transfected with E522K mutant, however, did not produce currents. Extracellular alkalinization from pH 7 to 10 did not bring out currents in E522K-transfected cells (not shown). Thus, the lack of currents for E522K is not due to a very alkaline p$K_a$ for the mutant. Further studies are required to understand the reasons for lack of currents for E522K mutant.

Fig. 1. Effect of extracellular acidification on TRPV5. A, solutions (concentration in mM) used for ruptured whole-cell recording. B, voltage protocol. C, I–V relationships of TRPV5 currents at different pH$e$ values. D, inward currents (in nA at –80 mV) at pH$e$ from 8.4 to 4.4 (shown by horizontal bars in both acidifying and alkalinizing order). LaCl$_3$ (1 mM, labeled as ‘La’$^+$) was added to determine leak currents. E, relationships of inward currents versus pH$e$. Inward currents (after subtraction of residual currents in the presence of La$^{3+}$) were normalized to maximal current at pH 8.4. Normalized currents (I/I$_{max}$ vs pH$e$) at different pH$e$ values (x axis) were fitted with a modified Hill equation using the Sigma-Plot program (18). p$K_a$ is the pH for 50% inhibition of the current. Data points (closed circles and error bars) represent mean ± S.E. For most of the data points, error bars are smaller than the symbols.
The amino acid glutamate 522 of rabbit TRPV5 is not conserved in human TRPV5 (14, 26). The human TRPV5 has the non-titratable glutamine at amino acid 522. We found that the \( pK_a \) for pH inhibition of human TRPV5 was not significantly different from that of E522Q mutant of rabbit TRPV5 (5.60 ± 0.2 versus 5.32 ± 0.1, not significant). Overall these results support the hypothesis that titration of amino acid at the position of glutamate 522 contributes to inhibition of TRPV5 channel by extracellular protons. As mentioned above, differences between apparent \( pK_a \) for pH inhibition of TRPV5 and that of free amino acids in solution are likely caused by local chemical environment (24, 25).
Fig. 4. Effects of substitution of different titratable amino acids for glutamate 522 on pH sensitivity. A, pK\text{a} values (n = 4–8 for each mutant, n = 32 for wild type) for inhibition of wild type and TRPV5 mutants (as indicated) versus pH. The experimental paradigm is as in Fig. 1. C, pK\text{a} values (n = 4–8 for each mutant, n = 32 for wild type) for inhibition of wild type and TRPV5 mutants (as indicated) by extracellular acidification. * indicates p < 0.05 versus wild type, ** indicates p < 0.05 versus E522Q mutant. Mean current density (pA/pF, mean ± S.E.) was 1,238 ± 213, 1,319 ± 381, 1,119 ± 199, and 1,183 ± 217 for E522Y, E522C, E522H, and E522D, respectively (not significant between wild type and each of the mutants). WT, wild type.

Effects of Extracellular Acidification on Single Channel Conductance of TRPV5—Single channel conductance of TRPV5 was measured by cell-attached single channel recording. At extracellular (pipette) pH 7.4, unitary inward current amplitude decreased as negative pipette potential (V\text{p}) was reduced from −120 to −60 mV (Fig. 5A). The unitary current-voltage relationship was linear over −120 to −60 mV, predicting a chord conductance (γ) of 91 ± 7.5 picosiemens (Fig. 5B). The effects of pH on single channel conductance were examined by cell-attached recording using different pipette pH values. At pH 8.4, single channel conductance (104 ± 45 picosiemens) was higher than at pH 7.4. At pH 6.4 and 5.4, conductances were reduced to 80 ± 5.1 and 58 ± 2.6 picosiemens, respectively. Compared with the reduction in whole-cell current, the reduction in single channel conductance by extracellular acidification was much smaller (78% reduction in whole-cell current versus 40% reduction in single channel conductance by acidification from pH 8.4 to 5.4, p < 0.05; Fig. 5C). Assuming no changes in the number of channels, reduction of whole-cell currents is due to reduction in single channel conductance and/or open probability. The above results thus suggest that extracellular acidification decreases both single channel conductance and open probability of TRPV5. As will be shown below in Fig. 5, E and F, only reduction in open probability is mediated by glutamate 522.

We next examined the effects of extracellular acidification on single channel conductance on E522Q mutant. We found that single channel conductance was lower in E522Q mutant relative to wild type (Fig. 5D). Extracellular acidification also decreased the single channel conductance on E522Q by an extent similar to that on wild type (Fig. 5E). In contrast to that for wild type, the percentage of reduction in single channel conductance and in whole-cell current by extracellular acidification was the same for E522Q (Fig. 5E). Fig. 5F shows the percentage of single channel conductance (gray bar) and whole-cell current (black bar) at pH 6.4 relative to pH 8.4 for wild type TRPV5 and for E522Q mutant, further illustrating differences in the effect of extracellular acidification for the two constructs. These results suggest that titration of glutamate 522 of TRPV5 by protons is responsible for decrease in open probability but not in single channel conductance of the channel. The mechanism and molecular determinant(s) responsible for acid-induced decrease in single channel conductance of TRPV5 require future investigation.

Effects of pH, on Wild Type and E522Q Mutant Channels in the Absence of Intracellular and Extracellular Mg\textsuperscript{2+}—Both intra- and extracellular Mg\textsuperscript{2+} cause voltage-dependent block to TRPV5 (27, 28). The molecular determinant for intracellular Mg\textsuperscript{2+} block is not known. The extracellular Mg\textsuperscript{2+} causes voltage-dependent block of TRPV5 by binding to aspartate 542 in the putative pore region (29). To see whether extracellular acidification may alter conformation of intracellular and/or extracellular Mg\textsuperscript{2+} binding site(s) to increase Mg\textsuperscript{2+}-mediated inhibition of the channel, we removed Mg\textsuperscript{2+} from both sides of the membrane by including 1 mM EDTA in the bath solution and 10 mM EDTA in the pipette solution in whole-cell recording (Fig.
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was increased from 1.36
 rents. The rectification ratio (ratio of inward/outward current
conductance (\(e^{100 \text{mV over 400 ms applied every 15 s}}\)). At pH 7.4,
 8.4 and 5.4 were normalized to that at pH 8.4. D, single channel conductance of wild type and E522Q mutant at different pH values. E, pH
sensitivity of whole-cell current and of single channel conductance for E522Q mutant. The curve of pH sensitivity of whole-cell current for E522Q is from Fig. 2.
Single channel conductance was normalized as in C, F, normalized percentage of whole-cell current (black bar) and single channel conductance (gray bar) at pH 6.4 versus at pH 8.4 for wild type and E522Q mutant. Data shown for wild type and E522Q are from Fig. 5, C and E, respectively. NS: not significant.

6A). I-V relationships recorded by voltage steps immediately after formation of ruptured whole-cell recording show the characteristic strong inward rectification of TRPV5 (Fig. 6B, inset). Rectification of currents became less apparent over \(-20 \text{ s}\) as EDTA in the pipette solution diffused inside cells (see I-V by voltage ramps at pH 8.4 in Fig. 6B). After currents stabilized, the effects of pH on I-V relationships were examined by voltage ramps (step to \(-100 \text{ mV for 50 ms and ramp from \(-100 \text{ to 100 mV over 400 ms applied every 15 s}}\)). At pH 8.4, the I-V relationship was closer to linear (Fig. 6B). Current reversed at \(16 \pm 3 \text{ mV}\). Extracellular acidification from 8.4 to 4.4 reduced currents to \(<10\%\) of the maximum (Fig. 6B). Extracellular acidification enhanced inward rectification of currents. The rectification ratio (ratio of inward/outward current at \(-80 \text{ mV and } +80 \text{ mV of reversal potential, respectively})
increased from \(1.36 \pm 0.08 \text{ at pH 8.4 (maximal current)}
\)
to \(2.59 \pm 0.32 \text{ at pH 6.4 (near } pK_a)\) \((n = 5 \text{ for each, } p < 0.05)\). The \(pK_a\) for inhibition of inward current by extracellular acidification \((6.43 \pm 0.15, \text{Fig. 6D})\) was not significantly different from that in the presence of Mg\(^{2+}\) \((6.55 \pm 0.05, \text{Fig. 1E})\).

We next examined the effect of extracellular acidification on E522Q mutant in the absence of extra- and intracellular Mg\(^{2+}\). The reversal potential for E522Q at pH 8.4 \((15 \pm 4 \text{ mV, Fig. 6C})\) was not different from that for wild type \((16 \pm 3 \text{ mV, Fig. 6B})\). Compared with wild type, extracellular acidification caused a smaller decrease in currents on E522Q (Fig. 6C).

Acidification also enhanced inward rectification on E522Q. The rectification ratios were \(1.44 \pm 0.12 \text{ at pH 8.4 (maximal current)}\) and \(2.83 \pm 0.35 \text{ at pH 5.4 (near } pK_a)\) for E522Q \((n = 5 \text{ each, } p < 0.05)\). The \(pK_a\) for inhibition of inward currents of E522Q by extracellular acidification \((5.23 \pm 0.12, \text{Fig. 6D})\) was not significantly different from that in the presence of Mg\(^{2+}\) \((5.32 \pm 0.1, \text{Fig. 2E})\). Overall these results suggest that inhibition of TRPV5 resulting from proton titration of glutamate 522 is independent of Mg\(^{2+}\).

Effects of Sulfhydryl-reacting Reagents on E522C Mutant—
The membrane-impermeable sulfhydryl-specific methanethiosulfonate reagents react with the thiol group of cysteine \((19, 30)\). Whole-cell currents of E522C were recorded by voltage ramps \((-100 \text{ to 100 mV over 400 ms applied every s})\). Application of methanethiosulfonate ethylammonium (MTSEA, 20 \(\mu\text{M}\)) to the bath solution caused a fast inhibition of E522C currents (Fig. 7A). The rate constant for inhibition was estimated at \(-15,680 \text{ s}^{-1}\). After inhibition, currents from E522C did not recover by washing off MTSEA (Fig. 7B). Subsequent application of reducing agent dithiothreitol allowed currents to recover, consistent with the idea of covalent modification of cysteine by MTSEA reagents. Wild type TRPV5 currents were not inhibited by 1 mM MTSEA in the bath (see Fig. 7D), confirming that modification by MTSEA occurs at the introduced cysteine residue.
We next examined the effects of different MTS reagents on E522C. Methanethiosulfonate ethyltrimethylammonium (MTSET) has one positive charge like MTSEA but is significantly larger than MTSEA (width/length/height: 5.8/4.1/4.6 Å, Refs. 30 and 31). Methanethiosulfonate ethylsulfonate (MTSES) has a negative charge and is intermediate in size between MTSEA and MTSET (30, 31). The reaction rate constants for MTS reagents with free thiols (as in dithiothreitol or -mercaptoethanol) in solution were estimated at 40,000 M⁻ s⁻¹ for both MTSET and MTSEA and 4,000 M⁻ s⁻¹ for MTSES, respectively (31). We found that MTSET inhibited E522C currents with a rate similar to MTSEA (rate constant, 13,231 versus 15,680 M⁻ s⁻¹; not significant) (Fig. 7C).

The similar rate of inhibition by MTSEA and MTSET despite a large difference in the molecular size suggests that the amino acid 522 is not located in the narrow part of the ion permeation pathway. Compared with MTSEA and MTSET, the rate of inhibition by MTSES was lower (rate constant, 2,727 M⁻ s⁻¹; Fig. 7C). This order of inhibition of E522C currents by MTS reagents (MTSEA ~ MTSET > MTSES) is in good agreement with the rate for reaction of these reagents with free thiols in solution. The rate constant for inhibition of E522C approaches the reaction rate of the MTS reagents with free thiols in solution, suggesting that amino acid 522 resides on the surface of TRPV5.

The rate constant for MTS reagents to modify cysteine residues residing in the cytoplasmic vestibule and the pore region of ion channels is typically 2–3 orders of magnitude lower (≈10–100 m⁻ s⁻¹, Ref. 32). To allow for comparison with the reaction rate of MTSEA modification of the cysteine residue at position 522, we introduced a cysteine residue in two additional positions. One of these is glutamate 535, which is believed to reside in the narrow pore region (29). The other is histidine 509, which is believed to be either within the fifth transmembrane domain (26) or in the junction between the fifth transmembrane domain and the linker to the outer vestibule (Ref. 4 and see Fig. 2A also). We found that MTSEA (1 mM) had no effect on H509C (Fig. 7D), suggesting that the side chain of cysteine at amino acid 509 is not accessible to the extracellular aqueous solution. Low concentration of MTSEA (20 μM) had no effects on E535C (not shown). Higher concentration of MTSEA (1 mM) nevertheless inhibited E535C currents (Fig. 7D). The rate constant for MTSEA inhibition of E535C was estimated at ∼39 M⁻ s⁻¹, ∼400 times lower than that for inhibition of E522C. These results are consistent with the idea that glutamate 522 resides on the surface and glutamate 535 resides in the narrow pore region of the channel.

**DISCUSSION**

Regulation of ion channels by pH may occur via direct proton titration of the channel or may be mediated by other molecules. In the present study, we report that extracellular protons inhibit TRPV5 by titrating glutamate 522 in the extracellular loop between the putative fifth transmembrane domain and the pore region. The importance of titration of glutamate 522 in sensing and mediating acid-induced inhibition of TRPV5 is evidenced from a decrease in pHₜₐₙ sensitivity by mutation to glutamine. The ability of other titratable amino acids to sub-
titrate for glutamate 522 in conferring high pH sensitivity further supports this conclusion.

Titration of amino acids by protons can cause changes in protein conformation. Several lines of evidence suggest that titration of glutamate 522 decreases TRPV5 activity by altering protein conformation. First, it decreases open probability of the channel. Second, amino acids with different charges (see Fig. 4A) and a wide range of molecular sizes (Van der Waals volume: 141, 118, and 86 Å³ for side groups of tyrosine, histidine, and cysteine, respectively; Ref. 33) can substitute for glutamate (Van der Waals volume: 109 Å³). These findings suggest that glutamate 522 is not likely located in the narrow region of the pore. Superficial location of the residue is further supported by studies using the substituted cysteine accessibility method. These findings suggest that the decrease in activity of the channel caused by titration of glutamate 522 is due to conformational changes of channel proteins.

Another consequence of titration of glutamate is neutralization of its negative charge. Negative charges in the extracellular vestibule may be important in maintaining a high concentration of permeating cations (34). Inhibition of voltage-gated Na⁺ channels by external protons is reported to be due to voltage-dependent block by intracellular Mg²⁺ and polyamines (35). Previous studies have reported that removing intracellular Mg²⁺ by EDTA decreases but does not completely eliminate rectification of TRPV6 currents (28), suggesting that other mechanism(s) (such as block by polyamines) may also be involved. It is interesting that in our study the I-V relationship for TRPV5 becomes closer to linear with 10 mM EDTA in pipette and an alkaline extracellular pH. Extracellular acidification enhances inward rectification of the channel. It has been reported that extracellular pH affects polyamine-mediated rectification for Kir6.2 via titration of a histidine residue in the cytoplasmic carboxyl-terminal domain (36). The mechanism by which extracellular pH affects rectification of TRPV5 will be an interesting subject for future investigation.

The amino acid glutamate 522 of rabbit TRPV5 is not conserved in human TRPV5 (14, 26). In human TRPV5, the equivalent amino acid is a non-titratable residue glutamine. Nevertheless the pH sensing mechanism mediated by the amino acid 522 is likely also important for pH regulation of epithelial Ca²⁺ transport in human. TRPV6, an isoform of TRPV5 also present in kidney and intestine, has a titratable histidine (conserved among species) at the position equivalent to the amino acid 522 of rabbit TRPV5 (26). As predicted from the histidine substitution mutant, TRPV6 is likely as sensitive to pH as the rabbit TRPV5. Indeed we found that the pKᵦ for pH inhibition of mouse TRPV6 was not significantly different from that of

![Figure 7](image-url)

**Fig. 7. Effect of MTS reagents on various cysteine substitution mutants.** A, inhibition of E522C by MTSEA at pH₈.4. The voltage protocol is as in Fig. 1. The dotted line shows zero current level. MTSEA was added at time 0. Numbers 1–6 and 17 indicate time in s after addition of MTSEA. B, recovery of MTSEA-inhibited E522C currents by dithiothreitol but not by washout of MTSEA alone. Inward currents (~80 mV) were normalized to current at time 0 (before application of MTSEA). C, rate of inhibition of E522C by different MTS reagents. D, effect of MTSEA on E535C, H509C, and wild type channel. DTT, dithiothreitol.
rabit TRPV5 (not shown). Hoenderop et al. (37) recently reported that TRPV5 and TRPV6 form heteromers and that heteromers have mixed biophysical and pharmacological properties of TRPV5 and TRPV6. It is likely that functional Ca\(^{2+}\) channels in human kidney and intestine are heteromers of TRPV5 and TRPV6. The histidine residue from TRPV6 will contribute to pH sensing for the heteromultimeric TRPV5/6 channels.

Metabolic acidosis increases urinary Ca\(^{2+}\) excretion (13). High dietary animal protein intake decreases urinary pH (by increasing acid load) and increases urinary Ca\(^{2+}\) excretion (38, 39). An increase in urinary Ca\(^{2+}\) predisposes individuals to formation of kidney stones (40). Administration of alkali in normal human subjects decreases urinary Ca\(^{2+}\) excretion (41).

How extracellular and/or intracellular acid increases urinary Ca\(^{2+}\) excretion in metabolic acidosis and in high dietary protein intake is not known. The pH of luminal fluid in DCT is in the range of 6–7 (21). The amount of Ca\(^{2+}\) excreted by the kidney is ~2% of the total filtered load. Of the 98% of the total filtered Ca\(^{2+}\) reabsorbed by the tubules, the DCT is responsible for ~15%. We found that extracellular acidification from pH 6.4 to 6.0 causes ~16% reduction in activity for channels containing a titratable glutamate (as in rabbit TRPV5) or histidine (as in human TRPV5). Assume that Ca\(^{2+}\) channels are 1:1 heteromers of TRPV5/6 and have an intermediate pH sensitivity of 12% reduction in channel activity per 0.4 pH unit acidification. Extracellular acidification from 6.4 to 6.0 will increase urinary Ca\(^{2+}\) excretion from 2 to 3.8% (2% × 15%) of the total filtered load. These results support the hypothesis that inhibition of Ca\(^{2+}\) reabsorption in DCT by extracellular protons contributes to increase in urinary Ca\(^{2+}\) excretion in the types of metabolic acidosis associated with a low luminal pH and high dietary protein intake.

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