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Regulation of TRPV5 Single-Channel Activity by Intracellular pH

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Abstract The transient receptor potential channel TRPV5 contributes to the apical entry pathway for transcellular calcium reabsorption in the kidney. Acid load causes hypercalciuria in animals and humans. We have previously reported that intracellular protons directly inhibit TRPV5. Here, we examined the effects of intracellular pH on single-channel activity of TRPV5. We found that TRPV5 channels exhibit full and subconductance open states in excised inside–out patches of Chinese hamster ovary cells. The slope conductance values (Na^+ as a charge carrier, between -25 and -75 m*V*) for full and subconductance opening at intracellular pH 7.4 were 59 ± 6 and 29 ± 3 pS, respectively. Intracellular acidification caused a small decrease in single-channel conductance. Importantly, intracellular acidification decreased open probability for the full and subconductance states and increased probability for closing. To investigate how intracellular protons decrease open probability of the channel, we proposed a simple three-state model for open–subconductance–closed state transition and examined the effects of acidification on the respective forward and reverse rate constants. We found that intracellular acidification decreases opening of TRPV5 predominantly by promoting a transition from the subconductance to the closed state. Thus, intracellular acidification directly inhibits TRPV5 by causing a conformational change(s) leading to a decrease of open probability of TRPV5 as well as of the single-channel conductance.

Keywords TRPV5 · Intracellular pH · Open probability · Subconductance state · Chinese hamster ovary cell

Introduction

Calcium (Ca^{2+}) is an abundant divalent cation critical for many processes including intracellular signaling, bone mineralization, formation of blood clots and regulation of cell–cell interactions. The kidney plays an essential role in maintaining calcium homeostasis by regulating its excretion. Of the Ca^{2+} filtered by the glomerulus, $\sim$98% is reabsorbed by the tubule, leaving $\sim$2% excretion by the kidney. The reabsorption by renal tubules occurs via both paracellular and transcellular pathways (Friedman & Gesek, 1995; Hoenderop, Nilius & Bindels, 2002). The transcellular reabsorption, occurring in the distal nephron of the kidney, accounts for $\sim$15–20% of total reabsorption.

Transient receptor potential (TRP) channels are a superfamily of cation-permeable channels classified into TRPC, TRPV, TRPM, TRPP, TRPML, TRPN and TRPA subfamilies (Montell et al., 2002; Clapham, 2003; Huang, 2004). The TRPV subfamily is named after the first mammalian member of the subfamily, vanilloid receptor 1 (VR1), and contains six mammalian members, TRPV1–6 (Jordt, McKemy & Julius, 2003). TRPV5 and TRPV6, cloned from kidney and intestine tissues, respectively (Hoenderop et al., 1999; Peng et al., 1999), are the only two highly Ca^{2+}-selective TRP channels (Clapham, 2003; Huang, 2004). The Ca^{2+}/Na^+ selectivity ratio of TRPV5 and TRPV6 is $\geq$100 (Vennekens et al., 2001a). TRPV5 and TRPV6 form heteromultimers, which likely constitute the

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apical Ca^{2+} channels that mediate *trans*-epithelial Ca^{2+} transport in kidney (Hoenderop et al., 2003).

Overall, cDNAs for TRP channels encode polypeptides of ~700–1,000 amino acids with amino acid homology. Hydrophobicity analysis of the TRP polypeptides including TRPV5 and TRPV6 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 Ca^{2+}-permeable channels and a carboxyl-terminal cytoplasmic terminus containing potential regulatory sites for protein kinases (Montell et al., 2002; Clapham, 2003; Huang, 2004).

The TRPV5/6 epithelial Ca^{2+} channel in the apical membrane of the distal nephron is likely the primary target for regulation of calcium homeostasis by hormones and acid-base disorders (Hoenderop, Nilius & Bindels, 2002). Acid load increases renal Ca^{2+} excretion, causing kidney stone formation (Sutton, Wong & Dirks, 1979; Breslau et al., 1988). Chronic metabolic acidosis decreases the expression of TRPV5/6 channels (Nijenhuis et al., 2006). For acute regulation by pH, we have reported that intracellular acidification inhibits whole-cell TRPV5 channel activity (Yeh et al., 2003, 2005; Yeh, Yoon & Huang, 2006). In the present study, we further investigate the biophysical basis of intracellular pH (pH_i) regulation of TRPV5 by analyzing its effect on single-channel properties.

Materials and Methods

Molecular Biology and Cell Culture

The cDNA for rabbit TRPV5 in a pCDNA3 mammalian expression vector has been described (Yeh et al., 2003, 2005, 2006). CHO-K1 clone cells (from ATCC, Rockville, MD) were cultured in F12-K medium (GIBCO, Grand island, NY) containing 10% fetal calf serum. Cells (at ~50% confluence) were cotransfected with cDNA for pEGFP plus cDNAs for TRPV5 using lipofectamine-plus transfection kits (GIBCO) and the protocol provided by the manufacturer's instruction manual. About 24–48 h after transfection, cells were dissociated by limited trypsin treatment and placed in a chamber for recording. Transfected cells were identified using epifluorescent microscopy (Yeh et al., 2003, 2005, 2006).

Patch-Clamp Recording

Single-channel recording of TRPV5 in inside–out patches was performed with an Axopatch 200B patch-clamp amplifier (Axon Instruments, Burlingame, CA) as described previously (Yeh et al., 2003, 2005, 2006). The pipette solution contained (in mM) 140 NaAsp, 10 NaCl, 10 ethylenediaminetetraacetic acid (EDTA) and 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.4). Membrane patches were excised into a bath solution containing (in mM) 140 NaAsp, 10 NaCl, 1 EDTA and 10 HEPES at different pH values as specified. Currents were low pass–filtered at 2 kHz using an eight-pole Bessel filter, sampled every 0.1 ms (10 kHz) with a Digidata-1300 interface (Molecular Devices, Sunnyvale, CA) and stored directly onto computer hard disk using pCLAMP9 software (Molecular Devices, Sunnyvale, CA). Data were transferred to CD for long-term storage. Single-channel current amplitudes and histograms were analyzed using the Clampfit9 program of pCLAMP9 software (Leung et al., 2000; Yeh et al., 2003, 2005, 2006). Open probability (P_o) was analyzed on segments of continuous recording (at least 5 min) from patches that contained only one active channel during the lifetime (>20 min) of the recording. P_o for full and subconductance openings in the same patch was determined using the criteria of threshold crossing of the current levels assigned for closed, subconductance and full states (i.e., baseline for closed, current level 1 for subconductance and current level 2 for full) (Leung et al., 2000). The current level for each state was assigned based on visual inspection of a long segment of recording for best fit and comparison with the current peaks in the amplitude histogram. For amplitude histogram analysis, only periods of recordings (5–30 s as specified in the figure legends) containing bursts of channel activity were used.

Data Analysis and Statistics

Data are shown as mean ± standard error of the mean (SEM) number of observations. Statistical comparison between two groups of data was made using two-tailed unpaired Student's *t*-test. Multiple comparisons were made using one-way analysis of variance followed by *t*-test. $P < 0.05$ was considered significant.

Results

We studied the regulation of TRPV5 single-channel activities by pH_i using Na^+ as a charge carrier and excised inside–out patch-clamp recordings (Fig. 1a). When extracellular and intracellular pH were both at 7.4, the single-channel P_o of TRPV5 was 0.35 ± 0.05 (mean ± SEM, $n = 6$; representative tracing in Fig. 1b). Both full and subconductance openings were observed when analyzed at an expanded time scale (bottom trace of Fig. 1b). Existence of

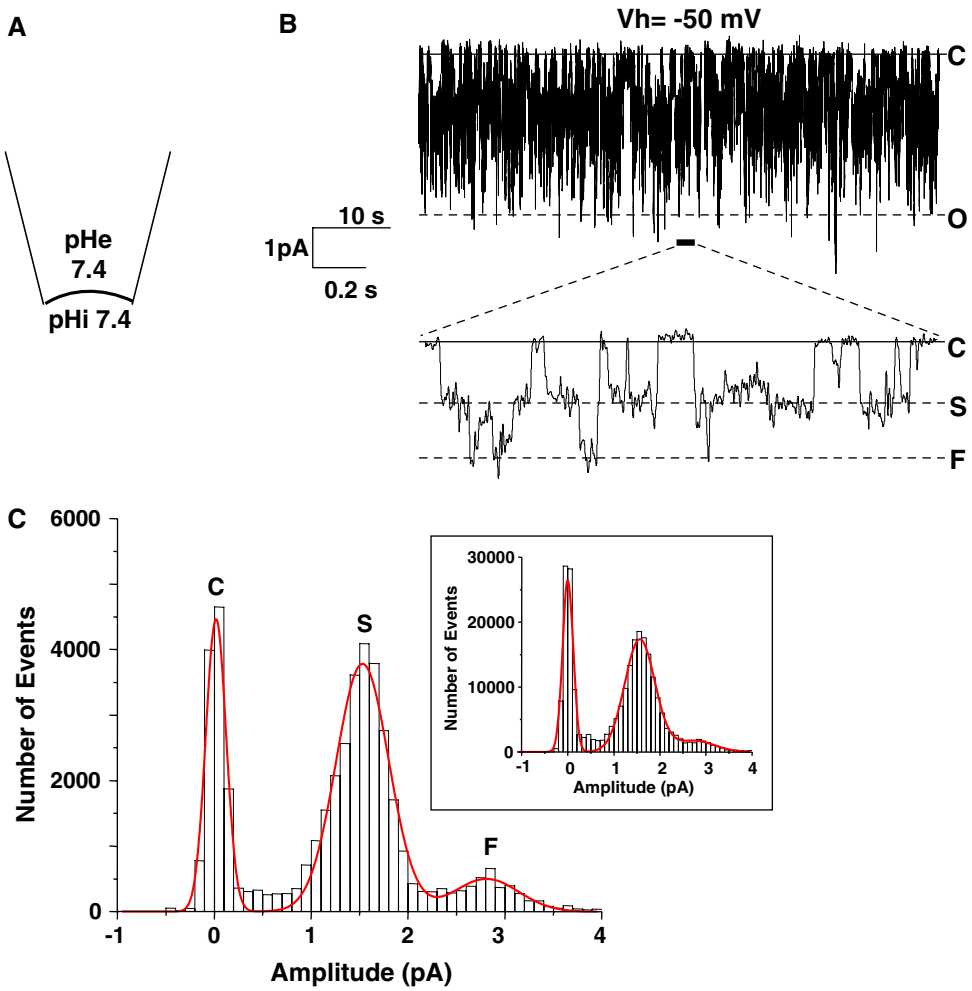

Fig. 1 Single-channel activity of TRPV5 at pH_i 7.4. **(a)** Condition of patch-clamp recording. **(b)** Single-channel recording at membrane holding potential (*Vh*) −50 m*V*. *Top* and *bottom tracings* represent compressed and expanded time bases, respectively. In the *bottom tracing*, *C, S* and *F* indicate current levels at closed, subconductance and full conductance opening, respectively. In the *top tracing*, *O* indicates current level at (full) opening. Scale bars for time duration (10 and 0.1 s) and current amplitude (1 pA) are shown. **(c)** Amplitude histogram of the recording from **b**. Bin width for amplitude histogram is 0.1 pA. Events of 5 s continuous recording were used for amplitude histogram analysis. *Inset* shows amplitude histogram from events of 30 s recording

the subconductance state was confirmed by amplitude histogram analysis (Fig. 1c). Of note is that TRPV5 opened at the subconductance (labeled "S") more frequently than at the full conductance state ("F") (Fig. 1c).

The single-channel current amplitude for full and subconductance states at −25, −50 and −75 m*V* membrane holding potential was measured (Fig. 2a). Membrane hyperpolarization from −25 to −75 m*V* did not affect single-channel open probability (*not shown*), indicating that gating of TRPV5 is not voltage-dependent. Figure 2b shows the relationship of current amplitude of the full and subconductance opening to membrane voltage (between −25 and −75 m*V*), which was used for calculation of the slope conductance. The average calculated slope conductance values of full- and subconductance open states (between −25 and −75 m*V* membrane holding potential and at pH_i 7.4) were 59 ± 6 and 29 ± 3 pS ($n = 6$ for each), respectively.

We next examined single TRPV5 channel activities at pH_i 7.0, 7.4 and 8.4 at a fixed extracellular pH of 7.4. Intracellular acidification from pH_i 7.4 to 7.0 decreased single-channel open probability of TRPV5 (P_o 0.35 ± 0.05 *vs.* 0.21 ± 0.07, $n = 5$, respectively; $P < 0.05$; representative tracings in Fig. 3a). Notably, opening at pH_i 7.0 is predominantly at the subconductance level. This finding is consistent with the notion that intracellular acidification decreases TRPV5 opening predominantly by favoring transition from the subconductance state to the closed state (*see* Fig. 6 and discussion below). Conversely, alkalinization to pH_i 8.4 increased open probability (P_o 0.35 ± 0.05 *vs.* 0.63 ± 0.08, $n = 5$, respectively; $P < 0.05$). Amplitude histogram revealed the existence of full and subconductance states and the relative distribution of closed (C), full open (F) and subconductance open (S) states at each pH_i (Fig. 3b). Consistent with the finding that alkalinization increases open probability, the relative distribution of full open and subconductance states *vs.* the closed state increased from pH_i 7.0 to 7.4 and to 8.4 (Fig. 3b).

To understand the biophysical mechanism by which pH_i regulates TRPV5 channel activity, we analyzed slope conductance (between −25 and −75 m*V*) of full and subconductance at different pH_i. Increasing pH_i from 7.0 to 7.4 did not affect the slope conductance of either the full or subconductance state (Fig. 4). Increasing pH_i to 8.4,

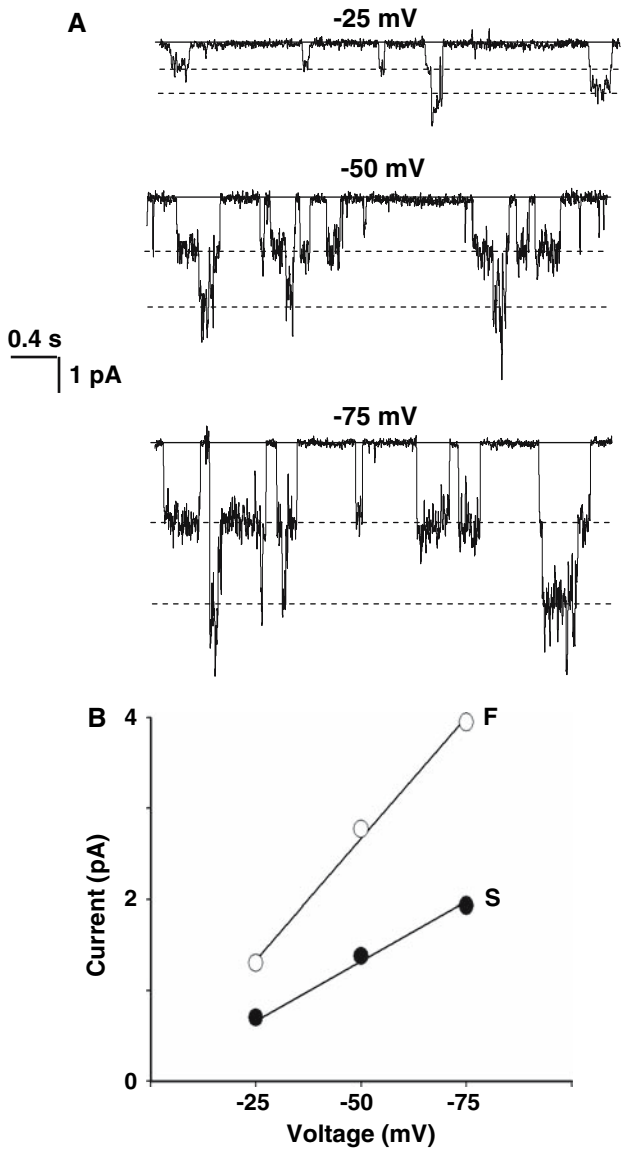

Fig. 2 Single-channel current amplitude of TRPV5 at different membrane holding potentials. **(a)** Current amplitude of subconductance and full conductance opening at −25, −50 and −75 m*V*. Scale bar of 1 pA is shown. **(b)** Relationship of current amplitude of full and subconductance states to membrane voltage (between −25 and −75 m*V*) from representative recording in **a**. *Open* and *closed circles* represent full and subconductance states, respectively

however, resulted in a small but significant increase of the slope conductance of both the full and subconductance states ($P < 0.05$, pH_i 8.4 *vs.* 7.0; Fig. 4). As mentioned above, increasing pH_i increased the overall open probability of TRPV5. The overall open probability measurement based on 50% threshold crossing includes events of opening at full conductance as well as subconductance states. Thus, we further analyzed the effects of pH_i on open probability of full and subconductance states separately (see "Materials and Methods"). As shown in Figure 5, subconductance opening of TRPV5 is more frequent than full conductance opening at pH_i ranging 7.0–8.4. Moreover, intracellular alkalinization of pH 7.0–8.4 increased opening at full conductance as well as in the subconductance state (Fig. 5). Consistently, the frequency of closed state decreased with intracellular alkalinization.

We proposed a three-state model to describe gating transition of TRPV5 (Fig. 6, see model on the left before addition of H^+). In this model, TRPV5 exists in one of the three states, closed ("C"), subconductance ("S") or full conductance ("F"). The subconductance state is between the closed and full open states. The transition between C and S states is governed by forward and reverse rate constants, K_1 and K_2, respectively (Hille, 2001). Likewise, the transition between S and F states is governed by forward and reverse rate constants, K_3 and K_4, respectively. The probability of a TRPV5 channel existing in the closed state (P_C) can be defined by forward and reverse rate constants K_1 and K_2 according to equation 1: $P_C = K_2/(K_1 + K_2)$. The probability of a TRPV5 channel existing in the full open state (P_F) can be defined by rate constants K_3 and K_4 according to equation 2: $P_F = K_3/(K_3 + K_4)$. The time constant (τ) for dwell time distribution of closed state (τ_C) is inversely related to K_1 according to equation 3: $\tau_C = 1/K_1$. The time constant for dwell time distribution of full open state (τ_F) is inversely related to K_4 according to equation 4: $\tau_F = 1/K_4$.

Figure 7 shows representative dwell time distributions of TRPV5 in closed and full open states at pH_i 7.4. As shown, the time constants for the representative dwell time distribution of TRPV5 in closed (τ_C) and full open (τ_F) states were 24 and 13 ms, respectively. To obtain all four rate constants, we first calculated K_1 and K_4 using τ_C and τ_F and equations 3 and 4, respectively. We then calculated K_2 and K_3 using values of probability of closed and full open states (from Fig. 5), time constants K_1 and K_4 and equations 1 and 2. Table 1 shows measured time constants for closed and full open state distribution and calculated kinetic rate constants of TRPV5 at pH_i 8.4, 7.4 and 7.0. As shown, intracellular acidification significantly increased K_2 and decreased K_3. No significant effects of intracellular acidification on K_1 and K_4 were observed. Based on these results, we propose that intracellular acidification favors TRPV5 entering the closed state by promoting the transition from subconductance state to closed state and decreasing the transition from subconductance to full open state (Fig. 6).

Discussion

We have shown that intracellular acidification inhibits TRPV5 channel activity in part by binding to and titration of a pH_i sensor, lysine-607 (Yeh et al., 2003, 2005, 2006).

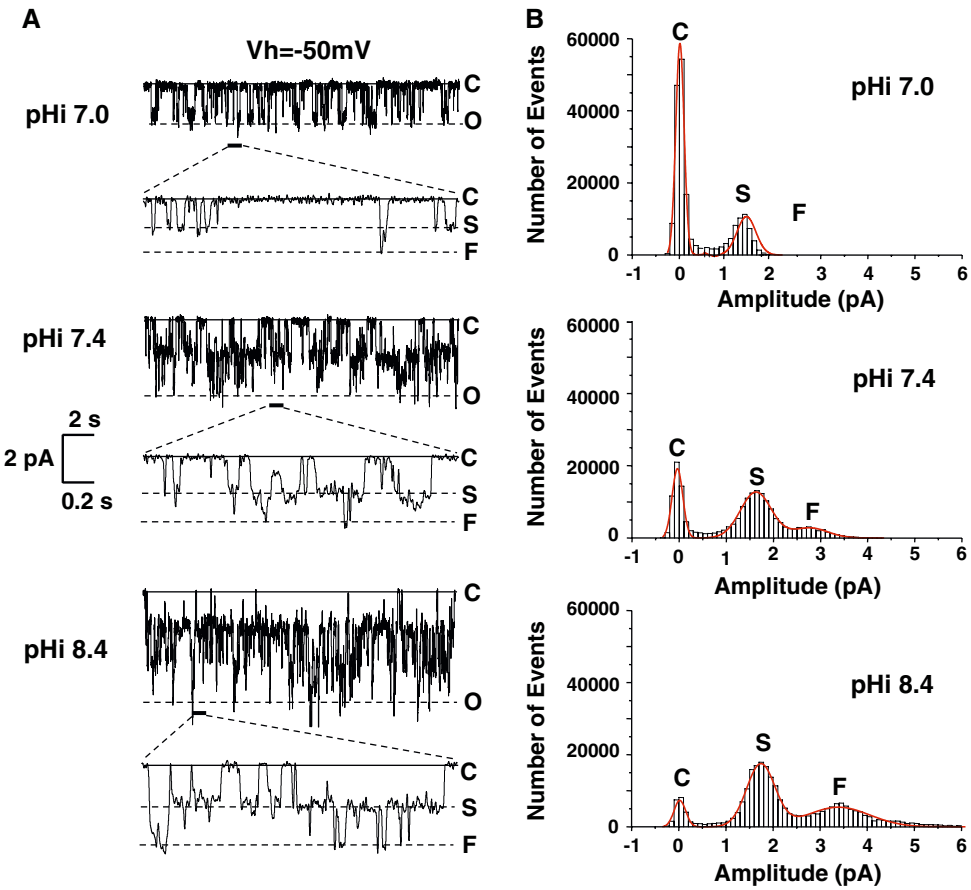

Fig. 3 Effect of pH_i on single TRPV5 channels. **(a)** Representative tracings of single TRPV5 currents at membrane holding potential (Vh) = −50 mV and different pH_i. **(b)** Representative amplitude histograms of recordings in **b**. Events of 30 s continuous recording were used for amplitude histogram analysis

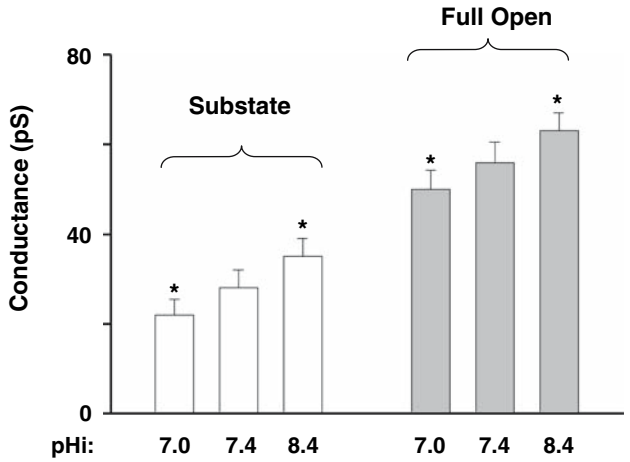

Fig. 4 Slope conductance of subconductance and full conductance opening of TRPV5 at different pH_i (mean ± SEM, n = 5, 6 and 5 for pH_i 7.0, 7.4 and 8.4, respectively). *Asterisks* indicate $p < 0.05$ vs pH 7.4

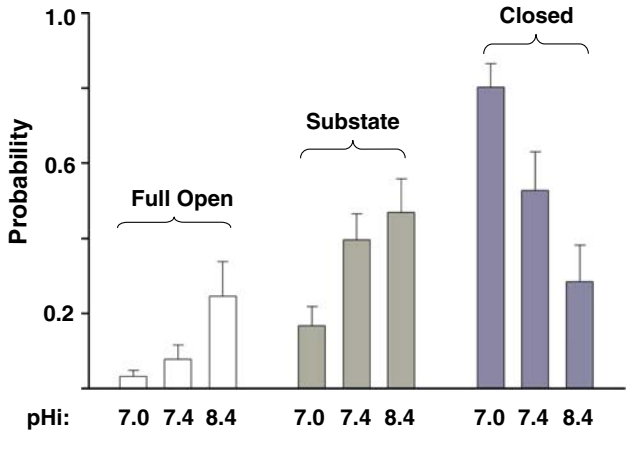

Fig. 5 Probability of full conductance, subconductance opening and closed state at different pH_i (mean ± SEM, n = 6 for each)

Binding of protons to channels can inhibit channel activity either by inducing conformational change(s) or by causing "proton block" (Hille, 2001). The proton-block mechanism occurs as a result of proton binding to the ion permeation pathway and causes an apparent reduction of single-channel current. Conformational changes occur as a result of proton binding to any part of channels and cause changes of open probability. Changes in single-channel conductance can also occur as a result of conformational changes of channel protein. Here, we show that intracellular acidification decreases open probability of TRPV5. A decrease in single-channel conductance also occurs but is relatively small compared to the decrease in open probability. These results support the hypothesis that binding of proton to a

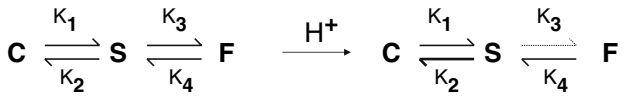

Fig. 6 A working three-state model of gating transition for TRPV5 in deprotonated (*left*) and protonated (*right*) states

pH_i sensor(s) causes conformational change(s) of the channel. The conformational change(s) favors the channel transitioning to the closed state. In addition, these changes impact ion permeation, decreasing single-channel conductance.

Another interesting finding of the present study is that TRPV5 exists in subconductance (a partial open) state when recorded using Na^+ as a charge carrier. The molecular nature of the partial open state is currently unknown. Opening of TRPV5 requires membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP_2) (Lee et al., 2005; Rohacs et al., 2005). Reduced interaction between PIP_2 and channel underlies subconductance opening of renal outer medullary k^+ channel (ROMK) (Leung et al., 2000). Future studies will investigate whether reduced interaction with PIP_2 underscores subconductance opening of TRPV5.

The physiological charge carrier for TRPV5 is Ca^{2+}. Because of very low single-channel currents, it is not practical to analyze single TRPV5 channel activity using Ca^{2+} as a charge carrier. Though we cannot exclude the possibility that the properties of single TRPV5 channel in Ca^{2+} solution are different from those in Na^+, it should be pointed out that the effects of pH on whole-cell TRPV5 Ca^{2+} currents are similar to those on Na^+ currents (Yeh et al., 2003, 2005).

Acid loading causes renal Ca^{2+} wasting (Sutton, Wong & Dirks, 1979; Breslau et al., 1988). It was reported that chronic intracellular acidosis decreases mRNA and protein abundance of TRPV5 in the distal nephron (Nijenhuis et al., 2006). It has also been suggested that acute intracellular acidification decreases surface abundance of TRPV5 by altering membrane trafficking of the channel (Lambers et al., 2007). The present study together with our previous study on whole-cell TRPV5 current suggest that acute inhibition of channel activity by intracellular protons is an additional mechanism for regulation of TRPV5-mediated Ca^{2+} reabsorption by pH_i in the kidney.

Extracellular acidification also inhibits TRPV5 (Vennekens et al., 2001b). Similar to that by intracellular acidification, the inhibition of TRPV5 by extracellular acidification occurs by decreasing open probability and single-channel conductance (Yeh et al., 2003). We have reported that extracellular and intracellular protons cross-regulate TRPV5, at least partly by affecting the same process, i.e., by causing a clockwise rotation of the pore helix (Yet et al., 2005). The findings that extracellular and intracellular protons have similar effects on single-channel properties are consistent with the notion that they regulate the same process. In clinical conditions of increased urinary calcium excretion from acid loading (e.g., high dietary protein intake), luminal (extracellular) acidosis occurs in addition to intracellular acidosis. The dual regulation of TRPV5 by extracellular and intracellular protons is likely

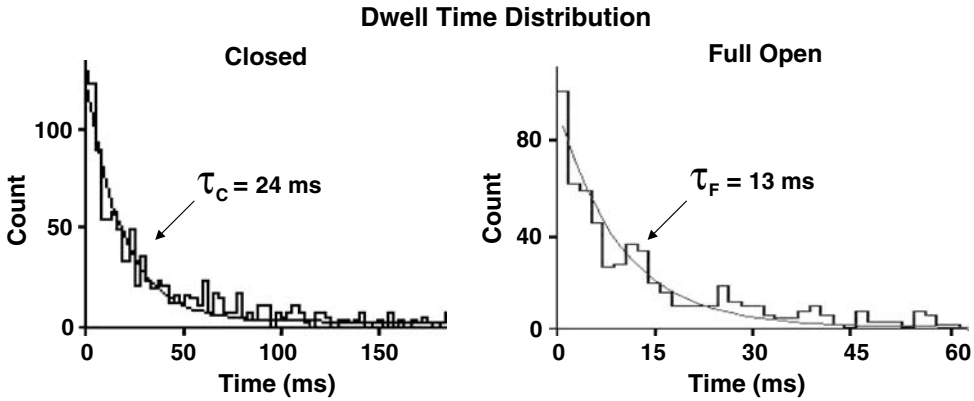

Fig. 7 Representative dwell time distributions for closed and full open states of TRPV5 at pH_i 7.4

Table 1 Effect of pH_i on full open (F) and closed (C) time distribution and the kinetic rate constants

pH_i	τ_F (ms)	τ_C (ms)	K_1 (s^{-1})	K_2^a (s^{-1})	K_3^a (s^{-1})	K_4 (s^{-1})
8.4	19 ± 4	19 ± 3	51 ± 7	20 ± 3	17 ± 2	52 ± 6
7.4	13 ± 2	24 ± 4	42 ± 6	47 ± 4	6 ± 1	74 ± 7
7.0	20 ± 3	29 ± 4	35 ± 6	140 ± 6	2 ± 1	50 ± 5

[a] Rate constants at pH_i 8.4, 7.4 and 7.0 were statistically significant different by analysis of variance

important for the effects of acid load on renal calcium transport.

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