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State-dependent Inhibition of TRPM2 Channel by Acidic pH*

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Transient receptor potential melastatin 2 (TRPM2) channel fulfills an important role in oxidative stress signaling in immune and other cells, to which local extracellular acidosis is known to occur under physiological or pathological conditions and impose significant effects on their functions. Here, we investigated whether the ADP-ribose-activated TRPM2 channel is a target for modulation by extracellular acidic pH by patch clamp recording of HEK293 cells expressing hTRPM2 channel. Induced whole cell or single channel currents were rapidly inhibited upon subsequent exposure to acidic pH. The inhibition in the steady state was complete, voltage-independent, and pH-independent in the range of pH 4.0–6.0. The inhibition was irreversible upon returning to pH 7.3, suggesting channel inactivation. In contrast, exposure of closed channels to acidic pH reduced the subsequent channel activation in a pH-dependent manner with an IC_{50} for H^+ of 20 μM (pH 4.7) and rendered subsequent current inhibition largely reversible, indicating differential or state-dependent inhibition and inactivation. Alanine substitution of residues in the outer vestibule of the pore including Lys^{952} and Asp^{1002} significantly slowed down or reduced acidic pH-induced inhibition and prevented inactivation. The results suggest that acidic pH acts as a negative feedback mechanism where protons bind to the outer vestibule of the TRPM2 channel pore and inhibit the TRPM2 channels in a state-dependent manner.

TRPM2[3] is member of the melastatin subfamily of transient receptor potential proteins (1–4) and forms Ca^{2+}-permeable cationic channels that are gated by intracellular ADPR or structurally related molecules (5–8). Substantial TRPM2 channel activation also occurs under oxidative stress. H_2O_2 opens the channels via ADPR-independent and or -dependent mechanisms (9–11). Intracellular Ca^{2+} strongly facilitates ADPR-induced channel activation or activates the channels (12–15). Previous studies have documented functional TRPM2 channels in neurons (16–19) and microglia in the brain (20–21), pancreatic β-cells (22–25), endothelial cells (26), and immune cells such as monocytes and lymphocytes (4, 8, 11, 27–33). Some of these studies have provided further evidence that TRPM2 channels mediate Ca^{2+} influx that is essential in production of cytokines and other inflammatory mediators by monocytes and increase in endothelial permeability in response to oxidative stress (26, 31, 32), and insulin secretion from pancreatic β-cells elicited by high levels of glucose (25). The functional role of the TRPM2 channels in the brain, despite abundant expression, is less clearly defined. Nonetheless, evidence has emerged to support that the TRPM2 channels may mediate neuronal death evoked by oxidative stress, amyloid β-peptide, and tumor necrosis factor-α, pointing to a potential role in the pathophysiology such as Alzheimer's diseases (16–17, 19, 34).

Extracellular acidification occurs around neurons undergoing intensive neuronal activity (35) and particularly at sites of infection and injury (36–38). It is unclear but important to know whether the TRPM2 channels present at such cellular settings are modulated by extracellular acidic pH, like many other ion channels (39–43). Here, we provide evidence that extracellular acidic pH imposes strong inhibition of the TRPM2 channel in a state-dependent manner.

MATERIALS AND METHODS

Clones, Cells, and Molecular Biology—The cDNA encoding the human TRPM2 (hTRPM2) was kindly provided by Dr. A. M. Scharenberg (University of Washington, Seattle, WA) (4). Human embryonic kidney (HEK) 293 cells were used to transiently express wild-type (WT) and mutant channels. Tetracycline-inducible HEK293 cells stably expressing the WT channel were used in some experiments. Cell culture, transfection, and induction were described previously (13–14). Mutations were introduced by site-directed mutagenesis and confirmed by sequencing. Chemicals and reagents used were purchased from Sigma except otherwise indicated.

Patch Clamp Recording—Whole cell and single channel current recordings were performed using an Axopatch 200B amplifier at room temperature as described previously (14, 44–46). Cells were held at −40 mV except otherwise stated. To record ADPR-induced currents, voltage ramps with 1-s duration from −120 mV to 80 mV were applied every 5 s. The currents at −80 mV denoted by *circles* in figures and the current-voltage (*I-V*) curves were obtained from the current responses to voltage ramps. In some experiments, cells were held constantly at −40 mV or 40 mV to record the inward and outward currents (see Fig. 2). Single channel recordings were carried out in outside-out configuration using glass pipettes of 8–10 MΩ and at −80 mV. Data were acquired at 4 kHz and filtered offline at 50 Hz. Intracellular solution contained 147 mM NaCl, 0.05

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[3] The abbreviations used are: TRPM2, transient receptor potential melastatin 2; ADPR, ADP-ribose.

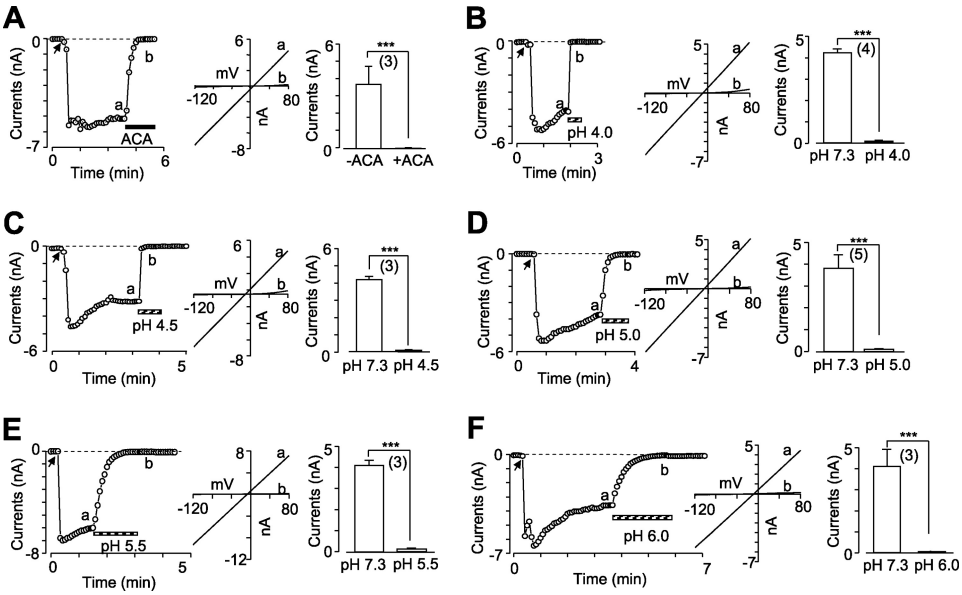

FIGURE 1. **pH-independent and irreversible inhibition by extracellular acidic pH of open TRPM2 channels.** ADPR-induced currents at −80 mV and the *I-V* relationship curves (at time points indicated by *a* and *b*) for WT TRPM2 channels were recorded using 1-s voltage ramps of −120 mV to 80 mV applied every 5 s, in pH 7.3 before and after application of 20 μM ACA (*A*)or initially in pH 7.3 and then in indicated acidic pH solutions (*B–F*). The *arrow* in each *panel* indicates the time point at which whole cell configuration was established. The numbers of cells examined in each case are indicated in *parentheses*. ***, $p < 0.001$ compared with the currents before and after application of ACA (*A*) or changes to acidic pH solutions (*B–F*). *Error bars*, S.E.

mM EGTA, 10 mM HEPES, 1 mM ATP, and 1 mM ADPR, pH 7.3. Extracellular solution contained 147 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES, and 13 mM glucose. The pH values of extracellular solutions were adjusted with concentrated HCl. The relative permeability of Na^+ and Ca^{2+} is close to 1 (46), and thus the currents were predominantly carried by Na^+ in the present study.

Two main protocols were used to study the effects of extracellular acidic pH. In the first protocol the patched cells were exposed to acidic pH after the currents were induced in pH 7.3 solution (*e.g.* see Figs. 1 and 5*A*). In the second protocol, immediately after cell-attached configuration was established in pH 7.3 solution, the patched cells were exposed to acidic pH for ≥2 min. In some experiments, the exposure to acidic pH continued for 1–2 min after whole cell configuration to allow dialysis of ADPR and channel activation in acidic pH solution (see Fig. 3). In some other experiments, exposure to acidic pH stopped immediately (<10 s) or 1 min before whole cell configuration and channel activation in pH 7.3 solution (see Fig. 4). For any residual currents at the end of each recording, N-(*p*-amylcinnamoyl)anthranilic acid (ACA) (20 μM) (Calbiochem) (47) or acidic pH 4.0 solution was applied. The cells, where the ADPR-induced currents showed complete inhibition by ACA or pH 4.0, were used in analysis. Changes of extracellular solutions and application of ACA were performed by using a RSC-160 system (Biologic Science Instruments) with a solution change time of ~300 ms (48).

Data Analysis—All results, where appropriate, are presented as mean ± S.E. The inhibition rate was estimated by determining the times required to reach 90% inhibition ($t_{90\%}$). The *smooth curve* in Fig. 3*C* represents the least squares fit to the Hill equation, $I/I_0 = 100/(1+([H^+]/IC_{50})^n)$, where I is the peak current in the indicated acidic pH solutions expressed as a percentage of the mean currents in parallel experiments using pH 7.3 solution, IC_{50} is the $[H^+]$ inhibiting half of the maximal currents, and n is the Hill coefficient. The steady-state whole cell currents in Fig. 6*D* in the indicated acidic pH solutions were expressed as percentage of the stable currents in pH 7.3 solution before solution change. Single channel events were displayed as all-point histograms, and single channel conductance were estimated based on the resolvable unitary currents as illustrated in Fig. 5, *A* and *C*. Curve fitting was performed using Origin software and statistical tests using Student's *t* test with $p < 0.05$ to be significant.

RESULTS

Effect of Extracellular Acidic pH on Open TRPM2 Channels—Whole cell recordings were made to measure ADPR-induced currents in HEK293 cells expressing hTRPM2 channel. ADPR (1 mM) applied via the intracellular solution induced currents of several nA at −80 mV with the typical TRPM2 channel properties, such as linearity of *I-V* curves and strong sensitivity to inhibition by ACA (Fig. 1*A*) as reported previously (14, 44–46). To test the potential for modulation of the TRPM2 channel by acidic pH, we first examined the effect of changing to pH 4.0–6.5 on the open channels. The currents that had been induced in pH 7.3 solution were inhibited rapidly and completely upon exposure to pH 4.0–6.0 (Fig. 1, *B–F*). The time required for the inhibition to reach steady state was progressively prolonged from <10 s in pH 4.0 solution to ≥120 s in pH 6.0 solution (Fig. 1, *B–F*). Unexpectedly, the currents were not recovered even after several minutes of reexposure to pH 7.3 (Fig. 1, *B–F*), with an exception of partial recovery from the effect of pH 5.5–6.0 observed in ~10% of cells. Exposure to pH 6.5 led to slow inhibition that failed to reach steady state over a period of >5 min, which prevented analysis because of concomitant nonspecific current run-down (data not shown). Nevertheless, the data suggest that open channels are profoundly inhibited by extracellular acidic pH.

Time courses or kinetics of inhibition were investigated for currents induced at constant membrane potentials of −40 mV or +40 mV. The kinetics of inhibition and the amplitude of the steady-state inhibition of pH 4.5 were similar for the inward and outward currents (Fig. 2*A*). In contrast, there were differential effects of pH 6.0 on inward and outward currents, with a prominent delay and slower effect on the outward currents despite no difference in the steady-state inhibition (Fig. 2*B*). The data indicate that the electric field or direction of ion permeation affects the action of moderate acidification. All the above data taken together suggest that extracellular acidifica-

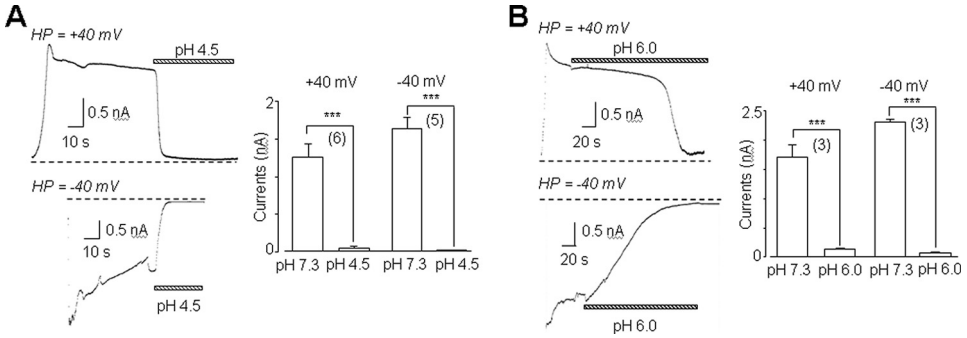

FIGURE 2. **Voltage-dependent effects by extracellular acidic pH on inward and outward TRPM2 channel currents.** ADPR induced currents for WT TRPM2 channels at constant holding membrane potentials (*HP*) of +40 mV (outward currents) or −40 mV (inward currents), first in pH 7.3 and then in pH 4.5 (*A*) or pH 6.0 (*B*). The *dotted lines* indicate the baseline. The numbers of cells examined in each case are indicated in *parentheses*. ***, $p < 0.001$ compared with the currents before and after exposure to the indicated acidic pH solutions. There is no significant difference in the steady-state inhibition between inward and outward currents and an obvious delay in reaching the steady-state inhibition of the outward currents in pH 6.0. *Error bars*, S.E.

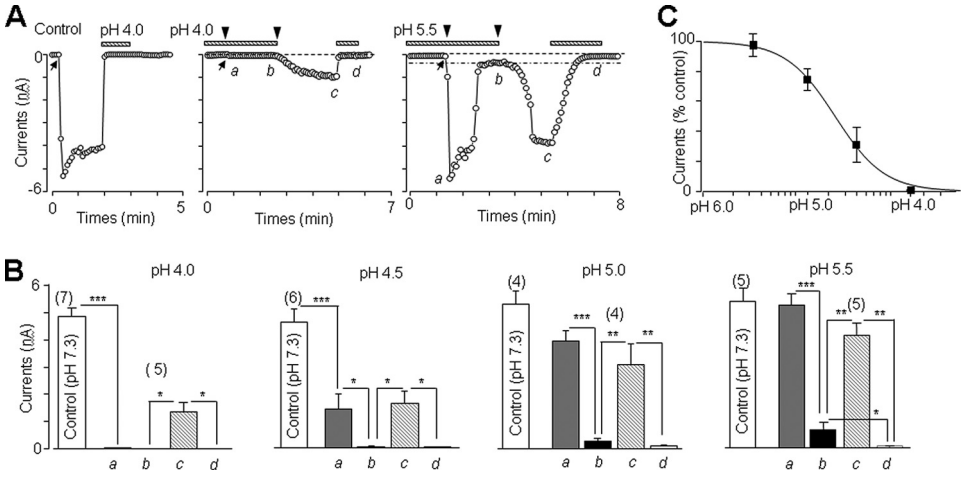

FIGURE 3. **TRPM2 channel activation and subsequent current inhibition in acidic pH solutions.** *A*, ADPR-induced currents at −80 mV for WT TRPM2 channels, using 1-s voltage ramps of −120 mV to 80 mV applied every 5 s. *Left*, control experiment showing that the currents induced in pH 7.3 were rapidly and irreversibly inhibited in pH 4.0. *Center*, cell exposed to pH 4.0 for 2 min (indicated by *two arrowheads*) after whole cell configuration. After the current recovery was completed upon returning to pH 7.3, the cell was reexposed to pH 4.0 followed by returning to pH 7.3. *Right*, similar recording made in pH 5.5, using the same protocols as in pH 4.0. The *arrow* in each recording indicates the time point at which whole cell configuration was established. The residual current in pH 5.5 is *highlighted* by the *dash-dot line*. *B*, summary of the currents in pH 4.0, 4.5, 5.0, or 5.5 at four time points denoted by *a*, *b*, *c*, and *d* shown in *A*. The numbers of cells recorded in each case are indicated in *parentheses*. ***, $p < 0.001$; **, $p < 0.005$; *, $p < 0.05$. *C*, pH dependence of channel activation in acidic pH solutions, using ADPR-induced currents at time point *a*, expressed as percentage of the mean currents in parallel control experiments shown in *B*. The *smooth curve* represents the least-squares fit to the Hill equation with an IC_{50} of pH 4.7 and Hill coefficient of 1.8. *Error bars*, S.E.

tion inhibits open TRPM2 channels and induces a conformational change in the channels leading to irreversible inactivation.

Effects of Extracellular Acidic pH on TRPM2 Channels in Closed State and Channel Activation—We next asked whether extracellular acidic pH affects closed TRPM2 channels and subsequent open channel activity. Such effects may have particular relevance to TRPM2 channels of lysosomal membranes where luminal (equivalent to extracellular) pH is highly acidic (24). After establishment of the cell-attached configuration in pH 7.3, channels were exposed to pH 4.0–5.5 for ≥2 min before breaking into whole cell configuration to allow dialysis of ADPR into the cells to activate the channels in the continuous presence of the acidic pH solution. As indicated in Fig. 3*A* and shown in *gray columns* in Fig. 3*B*, ADPR-induced currents immediately after breakthrough to the whole cell configuration (time point *a*) were undetectable in pH 4.0, strongly suppressed in pH 4.5, slightly suppressed in pH 5.0, and unaffected in pH 5.5 solution, revealing a graded pH-dependent effect on channel activation with an IC_{50} at pH 4.7 (H^+ concentration of 20 μM) (Fig. 3*C*).

Currents in pH 4.5–5.5 solutions were initially large and then quickly declined in the continuous presence of the acidic solutions (time point *b* in Fig. 3*A* and *black columns* in Fig. 3*B*). However, the inhibition was incomplete, as evidenced by residual current in pH 5.5 solution (denoted by the *dash-dot line* in Fig. 3*A*). Moreover, the inhibition was largely reversed upon returning to pH 7.3 (*cf. hatched* and *gray columns* in Fig. 3*B*). The recovered currents were completely and irreversibly inhibited upon reexposure to pH 4.0–5.5 (time point *d* in Fig. 3*A* and *unfilled columns* in Fig. 3*B*), as occurred in the experiments of Fig. 1, *B–F*.

In cells exposed to pH 4.0 for 2 min in the whole cell configuration, there was small recovery of current upon returning to pH 7.3 (Fig. 3*A*). We performed further experiments in which the exposure duration was shortened to 1 min or the cells were returned to pH 7.3 immediately after channel activation (<10 s) or 1 min before. As shown in Fig. 4, the irreversible inhibition was progressively reduced as the exposure duration was shortened and did not happen to the channels returned to pH 7.3 before activation. Thus, the channel inactivation depended strongly on the exposure duration to pH 4.0. This slower effect is in striking contrast with the fast inactivation (<30 s) of the open channels (Fig. 1*B*) or channels recovered in pH 7.3 solution (Figs. 3, *A* and *B*, and 4). The results suggest that binding of extracellular protons to the closed channels induces a conformational change that renders subsequently opened channels less prone to inactivation.

State-dependent Inhibition of Single TRPM2 Channels by Extracellular Acidic pH—To investigate inhibition of channels by acidic pH further, we performed experiments in excised outside-out membrane patches. The results are summarized in Fig. 5. Channel activity was first recorded in pH 7.3 solution. Acidifying the extracellular solution to pH 5.5 resulted in almost complete and irreversible channel inhibition (Fig. 5, *A* and *B*).

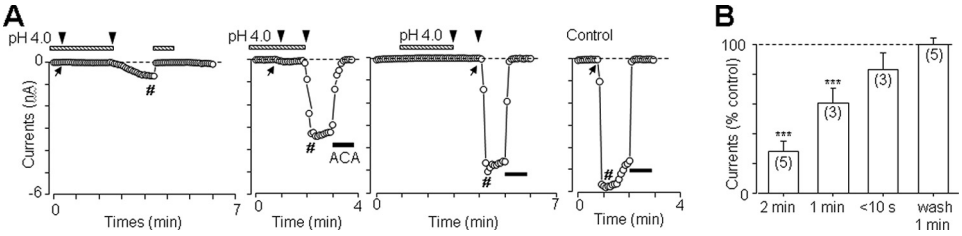

FIGURE 4. **Exposure time-dependent inactivation of TRPM2 channels preexposed to extracellular acidic pH in close state.** *A*, ADPR-induced currents at −80 mV for WT TRPM2 channels, using 1-s voltage ramps of −120 mV to 80 mV applied every 5 s. *Left*, a cell exposed to pH 4.0 for 2 min before whole cell configuration and continued to be exposed to pH 4.0 for another 2 min (indicated by *two arrowheads*) after whole cell configuration. When the current recovery was completed, the cell was reexposed to pH 4.0 followed by returning to pH 7.3. *Left center*, a similar recording in which the exposure time after whole cell configuration was shortened to 1 min. *Right center*, a similar recording in which the cell was returned to pH 7.3 for 1 min (indicated by *two arrowheads*) before whole cell configuration. *Right*, a control recording showing ADPR-induced currents in pH 7.3. The *arrow* in each recording indicates the time point at which whole cell configuration was established. *B*, summary of the maximal ADPR-induced currents recovered or induced in pH 7.3 (at time point denoted by # shown in *A*). The *column* denoted by *<10 s* shows the currents from cells that were returned to pH 7.3 immediately before whole cell configuration. The numbers of cells recorded in each case are indicated in *parentheses*. ***, $p < 0.005$ compared with parallel control experiments. *Error bars*, S.E.

Recordings made initially in pH 5.5 showed that there was recovery of channel activity upon exposure to pH 7.3 and irreversible inactivation upon subsequent reexposure to pH 5.5 (Fig. 5, *C* and *D*). The results are consistent with those from whole cell recordings. The single channel recordings also revealed that the unitary currents were reduced from 4 pA in pH 7.3 to 2.5 pA in pH 5.5 (Fig. 5), indicating that acidic pH suppresses the single channel conductance by ~40%.

Effects of Alanine Substitution of Candidate Proton-interacting Residues on Acidic pH-induced Inhibition and Inactivation of TRPM2 Channels—Histamine, glutamate, aspartate, and lysine residues are potential effectors for protons (42 and references therein). Eighteen such candidate residues are found in the hTRPM2 channel pore region, including Lys^{952} at the extracellular edge of the S5 (Fig. 6*A*). Each position was substituted with alanine. Fig. 6*B* shows the mean ADPR-induced inward currents for 10 of the mutants. R962A and R968A caused complete loss of function, and current amplitudes were significantly reduced for K952A, K1005A, and K1007A. The other mutants gave currents that were similar to those of the WT channel. Nine other mutants, including the nonfunctional E960A and D987A, which were previously reported (46), were further studied here in relation to acid sensitivity.

The 14 functional mutant channels were first exposed to pH 7.3 and then pH 4.5 or pH 5.5. The results are summarized in Fig. 6, *C* and *D*. As with the WT channel, the inhibition by pH 4.5 was rapid and exceeded 95%, although there was slightly smaller reduction for K952A, H973A, D1002A, and K1007A (*dotted bars* in Fig. 6*D*). There were more obvious differences when using pH 5.5, as illustrated in Fig. 6*C*. In cells expressing the K952A and D1002A mutant channels, substantial inhibition did not occur in response to pH 5.5 over several minutes. Residual currents were abolished by subsequent application of the TRPM2 blocker, ACA (Fig. 6*C*). On average, the residual currents expressed as percentages of the currents before exposure to pH 5.5 were 14 ± 2.8% and 39 ± 5.9% for K952A and D1002A, respectively (Fig. 6*D*); these values represent underestimates due to concomitant current rundown (Fig. 6*C*). On the basis of the time required to cause 90% inhibition ($t_{90\%}$), H958A (23 ± 6.4 s, $n = 7$; Fig. 6*C*), D964A (19.4 ± 3.5 s, $n = 4$; Fig. 6*C*), K1005A (11.9 ± 1.2 s, $n = 5$), and R1017A (15.9 ± 3.2 s, $n = 3$) accelerated, whereas H995A (106 ± 21 s, $n = 4$), E1010A (138 ± 12 s, $n = 5$; Fig. 6*C*), and D1012A (96 ± 15 s, $n = 5$; Fig. 6*C*) slowed down the inhibition relative to the WT channel (50 ± 4.5 s, $n = 9$; $p < 0.01$). Furthermore, the inhibition was partially reversible for K952A, E1010A, and D1012A and completely reversible for D1002A and E1022A (Fig. 6*C*). As summarized in Fig. 6*A*, these results suggest that several residues and particularly Lys^{952} and Asp^{1002} are important molecular determinants conferring on the TRPM2 channel the sensitivity to inhibition by extracellular acidic pH.

DISCUSSION

We investigated functional modulation of ADPR-induced TRPM2 channel currents by extracellular acidic pH and made three important findings. First, the TRPM2 channels in the open state are highly sensitive to inhibition by extracellular acidic pH. The steady-state inhibition was complete, voltage-independent, and pH-independent in the range of pH 4.0–6.0 (Figs. 1 and 2). The inhibition was also irreversible. We interpret these results to indicate that extracellular H^+ upon binding to the open channels can induce conformational changes leading to channel inactivation. As discussed later, this is most likely via interacting with the outer vestibule of the pore. Thus, the effect and mechanism bear some similarity with those previously reported for the TRPM5 (41) but completely differ from those for the TRPM6 and TRPM7 channels (39, 40, 49, 50).

During preparation of this manuscript for publication, two groups reported the effects of acidic pH mainly on the open TRPM2 channels (51, 52). All three studies consistently demonstrated strong inhibition but differ in four main aspects. The first one is exemplified by the reversibility of the inhibition or the acidic pH in which inactivation occurred: ≤pH 4.0 in the first study by Du *et al.* (51), ≤pH 5.0 in the second study by Starkus *et al.* (52), and ≤pH 6.0 in this study. This could be explained at least in part by the experimental conditions, including extracellular Ca^{2+} concentrations (52), channel (open or closed) states (Figs. 1, *B–F*, and 3, *A* and *B*) and exposure duration (Fig. 4*A*). The second difference is about the voltage dependence of the inhibition. The first study shows the steady-state inhibition to be voltage-independent (51). The second study, however, suggests that both the steady-state inhibition and the kinetics of inhibition are voltage-dependent, although the inhibition did not satisfactorily reach the steady state (Fig. 1, *C–E*, in Ref. 52). Our results from examining inhibition of the outward and inward currents (Fig. 2*B*) provide unambiguous evidence to indicate that the kinetics of inhibition is voltage-dependent but the steady-state inhibition is not. The apparent voltage dependence suggests to us differential influence of the direction of ion permeation on access and binding of

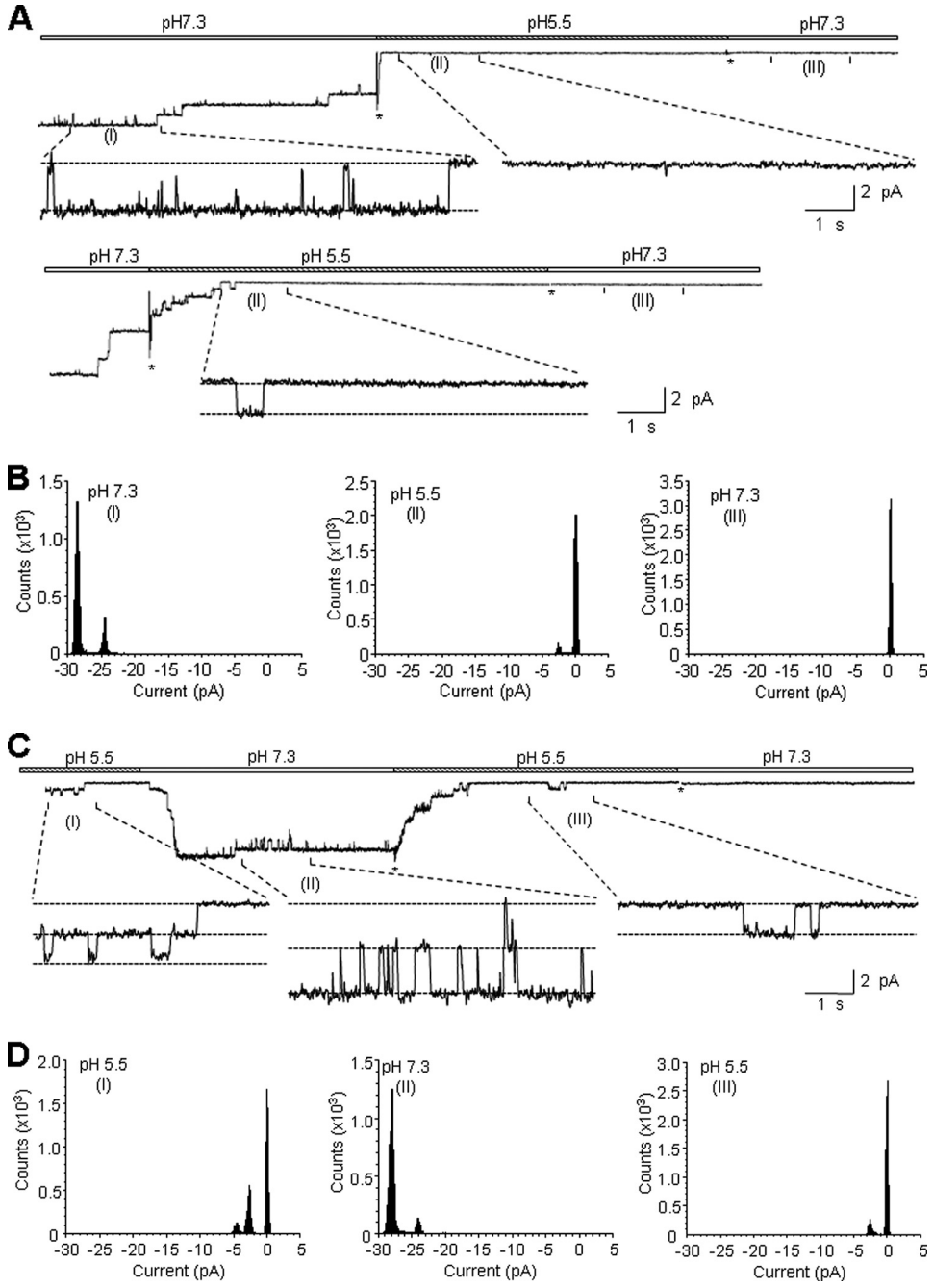

FIGURE 5. **State-dependent effects of extracellular pH 5.5 on TRPM2 single channel activities.** *A*, representative outside-out recordings of the effect of extracellular pH 5.5 on ADPR-evoked single channel currents previously induced in pH 7.3. Single channel events are clearly seen in the *expanded traces*, to which the scales are applicable. The resolvable unitary currents at −80 mV were ~4 pA and 2.5 pA in pH 7.3 and pH 5.5, respectively. The *asterisks* indicate the noises introduced by solution changes or a brief time gap in recording. *B*, all-point histograms of the current amplitudes from 10-s recordings in the indicated conditions as illustrated in *A*. *C*, representative outside-out recording of the effects of extracellular pH 5.5 on ADPR-evoked single channels, exposure of which to pH 5.5 started when the channels were in closed state (data not shown). Single channel events are clearly seen in the *expanded traces*, to which the *scales* are applicable. The resolvable unitary currents at −80 mV were ~4 pA and 2.5 pA in pH7.3 and pH5.5, respectively. The *asterisks* indicate the noises introduced by solution changes or a brief time gap in recording. *D*, all-point histograms of the current amplitudes from 10-s recordings in indicated conditions as illustrated in *C*.

extracellular H^+ to the open channels. The third difference is regarding the underlying mechanism. The first study concludes that extracellular H^+ inhibits the open channels via binding to the outer vestibule of the pore (51), whereas the second study supports the notion that extracellular H^+ permeates through, and inhibits intracellularly, the open channels (52). Our results and particularly those from site-directed mutagenesis as discussed later strongly favor an extracellular mechanism (51). The last difference is with respect to the effect of acidic pH on single channel conductance. The present study (Fig. 5), as did the first study (51), showed a reduction of ~40% in single channel conductance, whereas the second study found that the single channel conductance was reduced by ~35% for the native TRPM2 channels in neutrophils but was unaltered for the recombinant TRPM2 channel (52).

The second important finding from this study is strong and distinctive influence of extracellular acidic pH on channel activation and subsequent inhibition. In response to the same concentration of ADPR, no currents were detectable in pH 4.0, but the currents in pH 5.5 were not different from those in pH 7.3, resulting in a pH-dependent effect on channel activation, with an IC_{50} for H^+ of 20 μM (pH 4.7; Fig. 3*C*). Exposure of the closed channels to pH 4.0–5.5 had no effect on the currents subsequently induced in pH 7.3 (Fig. 4) or on the steady-state current inhibition by acidic pH, with an exception of pH 5.5 that prevented complete inhibition (Fig. 3, *A* and *B*). However, surprisingly, such exposure rendered the inhibition of the currents subsequently induced in acidic pH solutions to be highly reversible or the channel inactivation slower and less effective (Figs. 3, *A* and *B*, and 4). This strikingly contrasts with the fast (<30 s) and irreversible inactivation of the channels that were previously activated in pH 7.3 (Fig. 1) or activated in acidic pH solution but subsequently recovered in pH 7.3 (Fig. 3, *A* and *B*). These results were reproducible at the single channel level (Fig. 5). We suppose that all of the results indicate that extracellular H^+ binds to the closed channels in a reversible manner and that such binding makes the channels activated in acidic pH solution less prone to inactivation.

The third important finding is identification of several residues in the outer vestibule of the pore that are critically involved in the inhibition by extracellular acidic pH. For

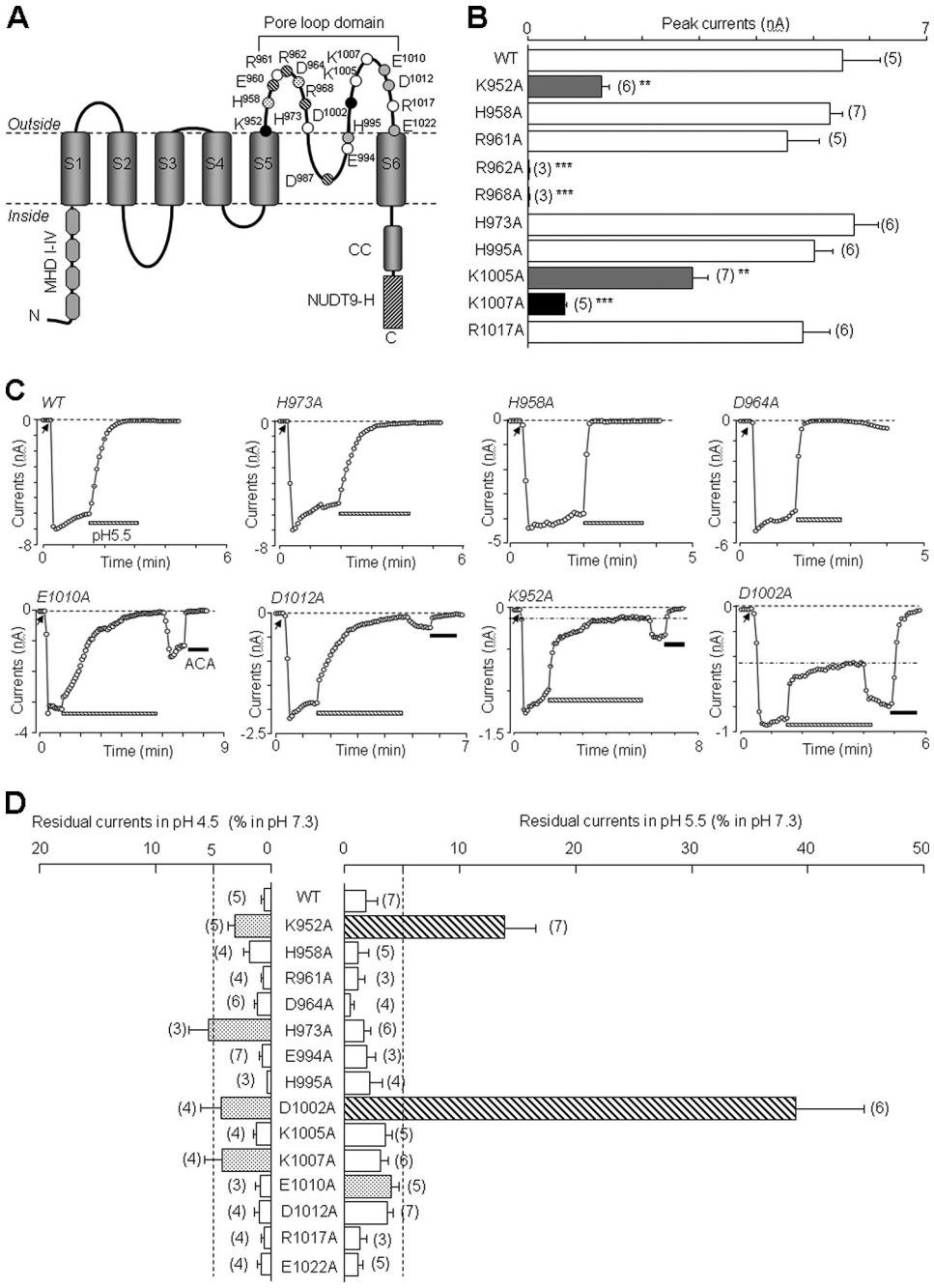

FIGURE 6. **Effects of alanine substitution of the pore residues on TRPM2 channel function and inhibition by extracellular acidic pH.** *A*, diagram of membrane arrangement of a TRPM2 channel subunit. *S1–S6*, six transmembrane segments; *P loop*, pore-forming domain; *MHD I–IV*, four TRPM homology domains; *CC*, coiled-coil required for TRPM2 channel assembly; *NUDT9-H*, NUDT9 homology domain for ADPR binding. The 18 positions investigated in this study are *highlighted*. Effects of alanine substitution are as follows (see *B–D*): complete of loss of channel function (Glu^{960}, Arg^{962}, Arg^{968}, and Asp^{987} in *hatched circles*), strong reduction in inhibition and reversible inhibition (Lys^{952} and Asp^{1002} in *black circles*), accelerated inhibition (His^{958} and Asp^{964} in *dotted circles*), and slowed down or reversible inhibition (His^{995}, Glu^{1010}, Asp^{1012}, and Glu^{1022} in *gray circles*). *B*, ADPR-induced currents for WT or the indicated mutants. The numbers of cells recorded in each case are indicated in *parentheses*. ***, $p < 0.001$; **, $p < 0.01$ compared with WT. *C*, representative ADPR-induced currents at −80 mV for WT or indicated mutants, using 1-s voltage ramps of −120 mV to 80 mV applied every 5 s. The recordings started in extracellular pH 7.3 and then in pH 5.5, before returning to pH 7.3. 20 μM ACA was applied to abolish residual and or recovered currents. The *arrow* in each panel indicates the time point at which whole cell configuration was established. The residual currents in pH 5.5 are *highlighted* by the *dashed lines* for K952A and D1002A. *D*, steady-state residual currents in pH 4.0 (*left*) or pH 5.5 (*right*), expressed as percentage of the currents in pH 7.3 before change to extracellular acidic solutions. The numbers of cells recorded in each case are indicated in *parentheses*. The *hatched bars* denote $p < 0.005$, and the *dotted bars* indicate $p < 0.05$, compared with WT. *Error bars*, S.E.

instance, mutation to alanine of Lys^{952} and Asp^{1002} strongly reduced the steady-state inhibition and partially or completely prevents channel inactivation (Fig. 6*C*-D). Significant but less dramatic effects resulted from mutation of His^{995}, Glu^{1010}, Asp^{1012}, and Glu^{1022} (Fig. 6*C* and "Results"). These residues are presumably not involved in Ca^{2+} and Na^+ binding because mutation of many of them had no effect on their permeability (46). Therefore, our results suggest that the inhibition by extracellular acidic pH results from H^+ interaction with the outer vestibule of the pore. This notion is consistent with the reduced single channel conductance (Fig. 5). The reduction in the sensitivity to inhibition by extracellular Ca^{2+} reported in the recent two studies (51, 52), and the slower and delayed inhibition of the Na^+-carrying outward currents by pH 6.0 (Fig. 2*A*) may reflect competitive binding for H^+ and Ca^{2+}, or Na^+ in the absence of Ca^{2+}, to the outer vestibule, because of close apposition of the Ca^{2+}-binding residues (*e.g.* Glu^{960} (46) with the H^+-binding residues identified here such as Asp^{1002}. One of the two recent studies also examined by site-directed mutagenesis 11 of the 18 residues we did here and found that none of their mutations reduced but H958Q, D964N, and E994Q increased the sensitivity to inhibition by extracellular acidic pH (51). In our study, H958A and D964A accelerated the inhibition (Fig. 5*A*). Thus, despite differing in detail, both studies implicate a possible role for His^{958} and Asp^{964}. The reasons for the major discrepancy in terms of mutational effects between two studies are unclear but could correlate with the different channel conformations manifested by the nature of inhibition, which was strongly reversible in the previous study (51) and completely irreversible in the present study.

We observed strong state-dependent effects by acidic pH both at the whole cell (*cf.* Figs. 1 and 3*A*) and single channel levels (Fig. 5). Fig. 7

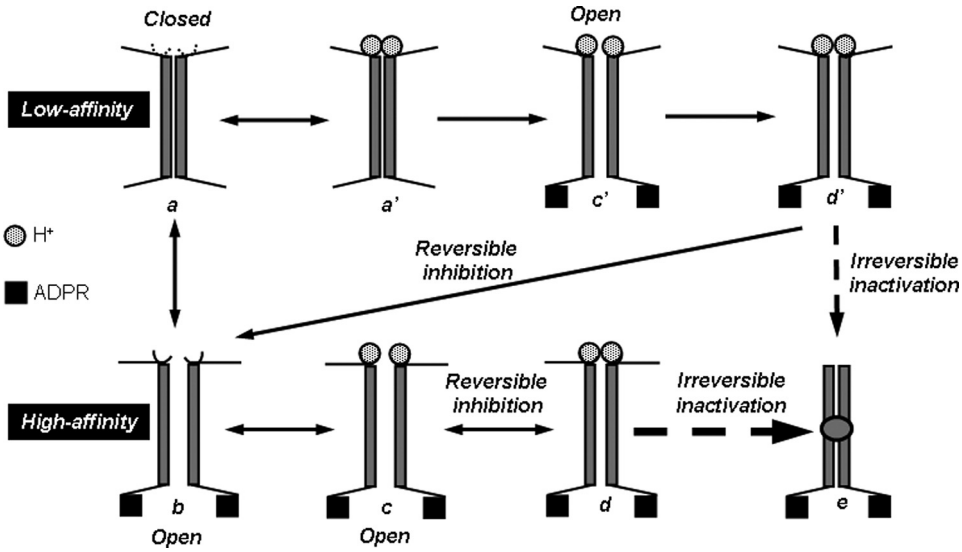

FIGURE 7. **Proposed mechanisms for state-dependent inhibition of TRPM2 channels by extracellular acidic pH.** H^+ binding sites in the outer vestibule of the pore exhibit low affinity (state *a*) when the channel is in closed state and increase or shows high affinity when the channel opens (state *b*). When binding to the high affinity sites (state *c*), extracellular H^+ reversibly inhibits the channels (state *d*) and induces rapid conformational changes leading to irreversible inactivation (state *e*). When binding to the low affinity sites (state *a'*), extracellular H^+ does not affect channel activation (state *c'*) and reversibly inhibits the channels (state *d'*) and induces inactivation, but preoccupation of the channels by extracellular H^+ imposes allosteric hindrance to the conformational changes required for inactivation and therefore makes H^+-bound open channels less prone to inactivation (state *e*). Those open channels that are recovered in pH 7.3 disclose the high affinity sites and, upon exposure to extracellular acidic pH, undergo the same reversible inactivation and irreversible inactivation processes (states *b*–*e*).

illustrates one simple and unifying scheme. Given that the residues contributing to the H^+ binding site we identified are located in the outer vestibule, it may be not totally unreasonable to assume that the H^+ binding sites in the closed channels show limited accessibility and lower affinity (state *a*). Conformational changes in the outer vestibule, accompanying channel opening in pH 7.3, increase accessibility and binding affinity of such sites (state *b*). Extracellular H^+, when binding to the high affinity sites (state *c*), results in reversible inhibition characterized by reduction in single channel conductance (state *d*) and elicits further conformational changes to inactivate the permeating pathway (state *e*) (Figs. 1, 2, and 5). On the other hand, when extracellular H^+ binds to the low affinity sites (state *a'*), the H^+-bound channels can open (state *c'*) and be reversibly inhibited by continuous exposure to acidic pH (state *d'*). However, H^+ binding imposes allosteric conformational hindrance to render the channels less prone to inactivation (state *e*), resulting in pH and exposure duration-dependent inactivation (Figs. 3–5). Once recovered from reversible inhibition upon returning to pH 7.3, the activated channels disclose the high affinity sites (state *b*) and undergo the same processes (states *c*–*e*) upon reexposure to acidic pH (Fig. 3), like those previously activated in pH 7.3 (Fig. 1). This scheme, despite being oversimplified, provides a useful framework for a mechanistic understanding of the modulation of the TRPM2 channels by extracellular acidic pH.

Extracellular acidosis develops at the sites of infection or injury and has well established effects on the function of immune and other cells (53). This study helps to understand how cells sense and respond to extracellular acidification. Increasing evidence supports a crucial role of the TRPM2 channels at the cell face in mediating oxidative stress and other signaling (5, 8, 30–32). Our findings suggest that extracellular acidification serves as a protective or negative feedback mechanism to limit oxidative stress-induced TRPM2 channel-mediated cytolytic effects. The state-dependent modulation bears strong implications for the TRPM2 channel functions, as the effects critically depend upon temporal occurrence of oxidative stress and extracellular acidification. TRPM2 also operates as a lysosomal Ca^{2+} release channel (24). The effects of highly acidic luminal pH on the TRPM2 channels are likely similar to those we showed here (Fig. 3). The luminal pH becomes less acidic (24), and thus the protective role of the luminal acidic pH is anticipated to be compromised during diseased conditions such as apoptosis.

In summary, we show that extracellular acidic pH imposes strong and state-dependent inhibition of the TRPM2 channels. Lys^{952} and Asp^{1002} and several other residues in the outer vestibule are important in mediating this inhibition. Such information helps us to understand better the mechanisms by which the TRPM2 channels are modulated under physiological and pathological conditions and the structure-function relationships of the TRPM2 channels.

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REFERENCES

1. Clapham, D. E. (2003) *Nature* **426,** 517–524
2. Fleig, A., and Penner, R. (2004) *Trends Pharmacol. Sci.* **25,** 633–639
3. Nilius, B., Owsianik, G., Voets, T., and Peters, J. A. (2007) *Physiol. Rev.* **87,** 165–217
4. Venkatachalam, K., and Montell, C. (2007) *Annu. Rev. Biochem.* **76,** 387–417
5. Perraud, A. L., Fleig, A., Dunn, C. A., Bagley, L. A., Launay, P., Schmitz, C., Stokes, A. J., Zhu, Q., Bessman, M. J., Penner, R., Kinet, J. P., and Scharenberg, A. M. (2001) *Nature* **411,** 595–599
6. Hara, Y., Wakamori, M., Ishii, M., Maeno, E., Nishida, M., Yoshida, T., Yamada, H., Shimizu, S., Mori, E., Kudoh, J., Shimizu, N., Kurose, H., Okada, Y., Imoto, K., and Mori, Y. (2002) *Mol. Cell* **9,** 163–173
7. Kolisek, M., Beck, A., Fleig, A., and Penner, R. (2005) *Mol. Cell* **18,** 61–69
8. Sano, Y., Inamura, K., Miyake, A., Mochizuki, S., Yokoi, H., Matsushime, H., and Furuichi, K. (2001) *Science* **293,** 1327–1330
9. Wehage, E., Eisfeld, J., Heiner, I., Jüngling, E., Zitt, C., and Lückhoff, A. (2002) *J. Biol. Chem.* **277,** 23150–23156
10. Perraud, A. L., Takanishi, C. L., Shen, B., Kang, S., Smith, M. K., Schmitz, C., Knowles, H. M., Ferraris, D., Li, W., Zhang, J., Stoddard, B. L., and Scharenberg, A. M. (2005) *J. Biol. Chem.* **280,** 6138–6148
11. Buelow, B., Song, Y., and Scharenberg, A. M. (2008) *J. Biol. Chem.* **283,**

24571–24583
12. Du, J., Xie, J., and Yue, L. (2009) *Proc. Natl. Acad. Sci. U.S.A.* **106,** 7239–7244
13. McHugh, D., Flemming, R., Xu, S. Z., Perraud, A. L., and Beech, D. J. (2003) *J. Biol. Chem.* **278,** 11002–11006
14. Mei, Z. Z., Mao, H. J., and Jiang, L. H. (2006) *Am. J. Physiol. Cell Physiol.* **291,** C1022–C1028
15. Starkus, J., Beck, A., Fleig, A., and Penner, R. (2007) *J. Gen. Physiol.* **130,** 427–440
16. Fonfria, E., Marshall, I. C., Boyfield, I., Skaper, S. D., Hughes, J. P., Owen, D. E., Zhang, W., Miller, B. A., Benham, C. D., and McNulty, S. (2005) *J. Neurochem.* **95,** 715–723
17. Hill, K., Tigue, N. J., Kelsell, R. E., Benham, C. D., McNulty, S., Schaefer, M., and Randall, A. D. (2006) *Neuropharmacology* **50,** 89–97
18. Olah, M. E., Jackson, M. F., Li, H., Perez, Y., Sun, H. S., Kiyonaka, S., Mori, Y., Tymianski, M., and MacDonald, J. F. (2009) *J. Physiol.* **587,** 965–979
19. Smith, M. A., Herson, P. S., Lee, K., Pinnock, R. D., and Ashford, M. L. (2003) *J. Physiol.* **547,** 417–425
20. Fonfria, E., Mattei, C., Hill, K., Brown, J. T., Randall, A., Benham, C. D., Skaper, S. D., Campbell, C. A., Crook, B., Murdock, P. R., Wilson, J. M., Maurio, F. P., Owen, D. E., Tilling, P. L., and McNulty, S. (2006) *J. Recept. Signal Transduct. Res.* **26,** 179–198
21. Kraft, R., Grimm, C., Grosse, K., Hoffmann, A., Sauerbruch, S., Kettenmann, H., Schultz, G., and Harteneck, C. (2004) *Am. J. Physiol. Cell Physiol.* **286,** C129–C137
22. Bari, M. R., Akbar, S., Eweida, M., Kühn, F. J., Gustafsson, A. J., Lückhoff, A., and Islam, M. S. (2009) *J. Cell. Mol. Med.* **13,** 3260–3267
23. Ishii, M., Shimizu, S., Hagiwara, T., Wajima, T., Miyazaki, A., Mori, Y., and Kiuchi, Y. (2006) *J. Pharmacol. Sci.* **101,** 174–178
24. Lange, I., Yamamoto, S., Partida-Sanchez, S., Mori, Y., Fleig, A., and Penner, R. (2009) *Sci. Signal.* **2,** ra23
25. Togashi, K., Hara, Y., Tominaga, T., Higashi, T., Konishi, Y., Mori, Y., and Tominaga, M. (2006) *EMBO J.* **25,** 1804–1815
26. Hecquet, C. M., Ahmmed, G. U., Vogel, S. M., and Malik, A. B. (2008) *Circ. Res.* **102,** 347–355
27. Gasser, A., Glassmeier, G., Fliegert, R., Langhorst, M. F., Meinke, S., Hein, D., Krüger, S., Weber, K., Heiner, I., Oppenheimer, N., Schwarz, J. R., and Guse, A. H. (2006) *J. Biol. Chem.* **281,** 2489–2496
28. Beck, A., Kolisek, M., Bagley, L. A., Fleig, A., and Penner, R. (2006) *FASEB J.* **20,** 962–964
29. Campo, B., Surprenant, A., and North, R. A. (2003) *J. Immunol.* **170,** 1167–1173
30. Heiner, I., Eisfeld, J., Halaszovich, C. R., Wehage, E., Jüngling, E., Zitt, C., and Lückhoff, A. (2003) *Biochem. J.* **371,** 1045–1053
31. Wehrhahn, J., Kraft, R., Harteneck, C., and Hauschildt, S. (2010) *J. Immunol.* **184,** 2386–2393
32. Yamamoto, S., Shimizu, S., Kiyonaka, S., Takahashi, N., Wajima, T., Hara, Y., Negoro, T., Hiroi, T., Kiuchi, Y., Okada, T., Kaneko, S., Lange, I., Fleig, A., Penner, R., Nishi, M., Takeshima, H., and Mori, Y. (2008) *Nat. Med.* **14,** 738–747
33. Zhang, W., Tong, Q., Conrad, K., Wozney, J., Cheung, J. Y., and Miller, B. A. (2007) *Am. J. Physiol. Cell Physiol.* **292,** C1746–C1758
34. Yamamoto, S., Wajima, T., Hara, Y., Nishida, M., and Mori, Y. (2007) *Biochim. Biophys. Acta* **1772,** 958–967
35. Chesler, M., and Kaila, K. (1992) *Trends Neurosci.* **15,** 396–402
36. Edlow, D. W., and Sheldon, W. H. (1971) *Proc. Soc. Exp. Biol. Med.* **137,** 1328–1332
37. Nielson, D. W., Goerke, J., and Clements, J. A. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78,** 7119–7123
38. Simmen, H. P., and Blaser, J. (1993) *Am. J. Surg.* **166,** 24–27
39. Jiang, J., Li, M., and Yue, L. (2005) *J. Gen. Physiol.* **126,** 137–150
40. Li, M., Du, J., Jiang, J., Ratzan, W., Su, L. T., Runnels, L. W., and Yue, L. (2007) *J. Biol. Chem.* **282,** 25817–25830
41. Liu, D., Zhang, Z., and Liman, E. R. (2005) *J. Biol. Chem.* **280,** 20691–20699
42. Liu, X., Ma, W., Surprenant, A., and Jiang, L. H. (2009) *Br. J. Pharmacol.* **156,** 135–142
43. Mao, J., Li, L., McManus, M., Wu, J., Cui, N., and Jiang, C. (2002) *J. Biol. Chem.* **277,** 46166–46171
44. Mei, Z. Z., and Jiang, L. H. (2009) *J. Membr. Biol.* **230,** 93–99
45. Mei, Z. Z., Xia, R., Beech, D. J., and Jiang, L. H. (2006) *J. Biol. Chem.* **281,** 38748–38756
46. Xia, R., Mei, Z. Z., Mao, H. J., Yang, W., Dong, L., Bradley, H., Beech, D. J., and Jiang, L. H. (2008) *J. Biol. Chem.* **283,** 27426–27432
47. Kraft, R., Grimm, C., Frenzel, H., and Harteneck, C. (2006) *Br. J. Pharmacol.* **148,** 264–273
48. Spelta, V., Jiang, L. H., Surprenant, A., and North, R. A. (2002) *Br. J. Pharmacol.* **135,** 1524–1530
49. Jiang, J., Li, M. H., Inoue, K., Chu, X. P., Seeds, J., and Xiong, Z. G. (2007) *Cancer Res.* **67,** 10929–10938
50. Numata, T., and Okada, Y. (2008) *J. Biol. Chem.* **283,** 15097–15103
51. Du, J., Xie, J., and Yue, L. (2009) *J. Gen. Physiol.* **134,** 471–488
52. Starkus, J. G., Fleig, A., and Penner, R. (2010) *J. Physiol.* **588,** 1227–1240
53. Lardner, A. (2001) *J. Leukocyte Biol.* **69,** 522–530