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Extracellular acidic pH-activated, outward rectifying chloride currents can be regulated by reactive oxygen species in human THP-1 monocytes

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ABSTRACT

Extracellular acidic pH-activated chloride channels ($I_{Cl,acid}$) have been found in a variety of mammalian cells. In the present study, the expression and regulation of $I_{Cl,acid}$ were investigated in THP-1 cells. Patch clamp recordings demonstrated that an extracellular acidic solution induced an outward rectified current, which could be blocked by the Cl^- channel blocker. The currents exhibited time-dependent facilitation and inactivation. The relative anion permeability of this current followed the sequence $Cl^- > Br^- > I^- >$ gluconate. NADPH oxidase inhibitors did not decrease pH 4.4-induced currents. However, reactive oxygen species (ROS) scavengers and mitochondrial inhibitors inhibited pH 4.4-induced currents. Fluorescence imaging of intracellular ROS and mitochondrial activity confirmed these findings. We conclude that $I_{Cl,acid}$ occurs in human THP-1 cells and that $I_{Cl,acid}$ may be regulated by intracellular ROS mainly originating from mitochondria.

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1. Introduction

THP-1 cell is a human monocytic leukemia cell line that represents a useful model to study the functional regulation of monocytes/macrophages [1]. Previous studies have suggested that ion channels play important roles in the regulation of differentiation and function in THP-1 cells [2,3]. Recently, an extracellular acidic pH-activated, outward-rectifying chloride channel ($I_{Cl,acid}$) has been identified in a variety of mammalian cells [4–6]. However, it is unclear whether $I_{Cl,acid}$ exists in THP-1 cells. Therefore, we first attempted to detect $I_{Cl,acid}$ in THP-1 cells.

Furthermore, the regulation of $I_{Cl,acid}$ remains largely unexplored. Most previous studies have found that the chloride transport inhibitor (4,4′-diisothiocyanostilbene-2,2′-disulphonic acid, DIDS) can inhibit $I_{Cl,acid}$[4–6]. Interestingly, Shi et al. found that $I_{Cl,acid}$ could be inhibited by simvastatin [7]. Previous studies have revealed that extracellular acidic pH is associated with increased amounts of intracellular reactive oxygen species (ROS) [8,9] and that ROS can regulate ion channels [10]. Therefore, we hypothesized that intracellular ROS signaling could regulate $I_{Cl,acid}$ in THP-1 cells.

2. Materials and methods

2.1. Cell culture

Human THP-1 cells (American Type Culture Collection) were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, 100 U/ml penicillin and 1.5% sodium bicarbonate at 37 °C, 5% CO_2 and 95% air.

2.2. Electrophysiology

THP-1 cells were subcultured onto glass coverslips for at least 1 h before patch clamping. Micropipettes were constructed from borosilicate glass capillaries with an outside diameter of 1.2 mm. The micropipettes were then fire-polished and filled with pipette solution, after which their resistance was 3–5 MΩ. Voltage clamping was performed at room temperature (23–26 °C) in whole-cell mode. Signals were amplified with an HEKA EPC-10 patch clamping amplifier and controlled with Pulse software (HEKA, Lambrecht, Germany). The voltage protocol used a 1-s ramp from −100 to +100 mV applied every 10 s with a holding potential of −60 mV. The current was sampled at −80 and +80 mV. To study the pH-dependence of $I_{Cl,acid}$, THP-1 cells were perfused with solutions of various pH (pH 7.4, 6.4, 5.4 and 4.4). To investigate the effect of various drugs on the currents, the cells were perfused with bath solutions at pH 7.4, 4.4 and 4.4 in the presence of the drugs.

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2.3. Measurement of intracellular ROS level

The production of intracellular ROS was monitored using dihydroethidium (DHE) as a fluorescent dye, which was oxidized to the fluorescent form, ethidium. THP-1 cells were treated with DHE (10 μmol/L) in the bath solution for 30 min at 37 °C. The fluorescence was monitored at the excitation and emission wavelengths of 488 and 510 nm, respectively, using an inverted microscope (Nikon TE2000-U, Nikon, Japan) and a TILLvisION digital imaging system (TILL Photonics GmbH, Munich, Germany) [11]. After incubation with various reagents, background intensity was subtracted from the fluorescent intensity changes, and the resulting values were then normalized as differences between the fluorescence intensities and the intensity at pH 7.4. Fluorescence values were determined by averaging the fluorescence values of at least 50 cells/treatment.

2.4. Measurement of mitochondrial activity using fluorescence imaging

The mitochondrial membrane potential was measured using rhodamine-123 (Rh-123) fluorescent imaging. Rh-123 is a cell-permeant, fluorescent dye that is readily uptaken by active mitochondria. THP-1 cells were loaded with Rh-123 (1 nM) in the bath solution for 15 min at 37 °C. Then, THP-1 cells were continuously perfused with bath solution. Rh-123 fluorescence images were captured as described above. The fluorescence was excited at 490 nm and fluorescence emission was measured at 530 nm. The background intensity was subtracted from the fluorescent intensity changes, and the resulting values were then normalized as the differences between the fluorescence intensities and the intensity at pH 7.4.

2.5. Reagents and solutions

The standard bath solution was comprised as follows (in mM): 130 NaCl, 10 CsCl, 1.2 $MgCl_2$, 1.5 $CaCl_2$, 10 HEPES and 10 glucose titrated to pH 7.4 with NaOH and adjusted to an osmotic pressure of 290 mOsm with D-Mannitol. For whole-cell patch-clamp recordings, the pipette solution contained (in mM) 140 CsCl, 1 $MgCl_2$, 20 EGTA and 10 HEPES titrated to pH 7.2 with CsOH. CsCl was used to block potassium channels, and Cl^- was the only anion component of the solutions. To study the anion selectiveness of $I_{Cl,acid}$, Cl^- was substituted by Br^-, I^- or gluconate. Stock solutions of DIDS, diphenyleneiodonium (DPI), DHE, and rhodamine 123 was prepared in dimethylsulfoxide (DMSO). N-acetyl-L-cysteine (NAC), 5-hydroxydecanoate (5-HD), tiron and gp91ds-tat were dissolved in distilled H_2O. All chemicals were obtained from Sigma (St. Louis, MO, USA) and diluted on the day of the experiment. The osmolarity of the solution was measured using a Micro-Osmometer 210

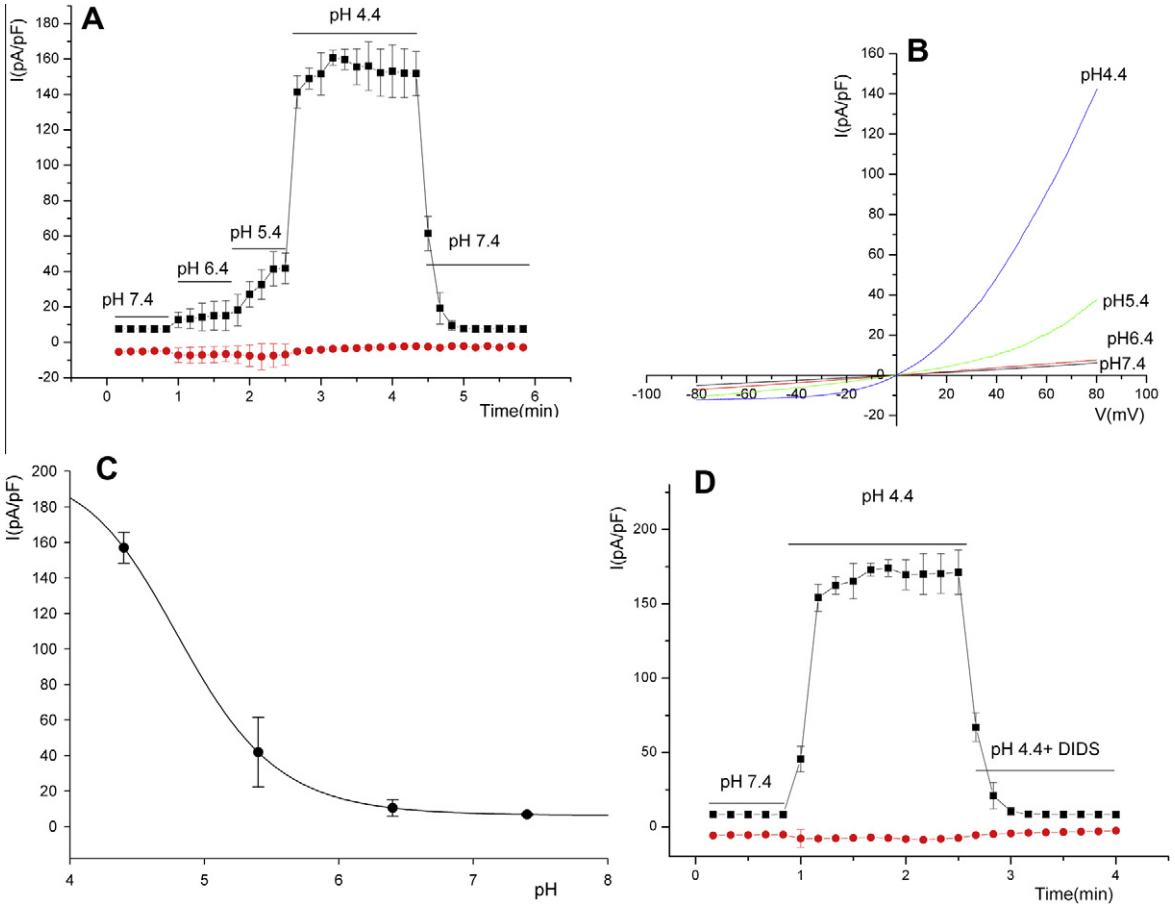

Fig. 1. Decreasing the extracellular pH from 7.4 to 4.4 induces an outward rectifying current in THP-1 cells (n = 10–12). (A) Time series of mean current recordings at membrane potentials of +80 and −80 mV obtained from voltage ramps during the application of solutions of various pH. The activations and deactivations of the current were rapid and repeated when the pH was changed from 7.4 to 4.4 or from 4.4 to 7.4. (B) Corresponding current–voltage relationships at the last sweep of every voltage ramp at various pHs. The outward current exhibited a clear outward rectification. (C) pH dependence of the outward current at a membrane potential of +80 mV in THP-1 cells. pH-response curves were fitted, and the following were calculated: EC_{50} (the pH at which a half-maximal response was induced) pH = 4.84, Hill coefficient nH = 13.51. (D) Mean current traces obtained at pH 4.4 (acidic conditions) in the absence or presence of the chloride channel blocker DIDS (100 μM). DIDS completely abolished the outward current activated at pH 4.4.

(Fiske, USA), and pH was monitored using a pH electrode connected to a pH meter (Thermo Orion 920A+, Thermo Scientific, USA). Neither the pH nor the osmolarity was affected by any of the drugs tested.

2.6. Data analysis

The data were expressed as means ± standard errors One-way ANOVA with repeated measures was used for statistical analysis. pH-response curves were fitted using the following equation: $I = a/(1 + EC_{50}/pH)^{nH}$), where a represents the amplitude of the $I_{Cl,acid}$ current, EC_{50} represents the pH at which a half-maximal response was induced, and nH is the Hill coefficient. The criterion of significance was set at $P < 0.05$.

3. Results

3.1. Extracellular acidic pH-activated, outward-rectifying chloride currents in human THP-1 cells

In human THP-1 cells, extracellular pH 4.4 solution induced a large outward current at positive holding potentials. The currents were induced and recovered quickly when the pH was changed from 7.4 to 4.4 or from 4.4 to 7.4 (Fig. 1A). The *I–V* relationship of the acidic pH-activated current exhibited a clear outward rectification (Fig. 1B) with a reversal potential of 0 mV. THP-1 cells were perfused with solutions at various pHs to obtain pH response relationships for acidic pH-induced currents at +80 mV (Fig. 1C). An analysis with the Hill equation revealed that the EC_{50} was pH 4.84.

The Cl^- channel inhibitor DIDS (100 μM) completely inhibited the outward current activated by an extracellular pH 4.4 solution (Fig. 1D).

3.2. Characteristics of acidic pH-induced currents

At depolarizing potentials, the currents induced by the pH 4.4 solution exhibited time-dependent facilitation and inactivation (Fig. 2A). *I–V* relationships exhibited that the outward current showed a clear outward rectification with a reversal potential of 0 mV (Fig. 2B). The relative anion permeability of this current followed the sequence $Cl^- > Br^- > I^- >$ gluconate. (Fig. 2C).

3.3. Effect of a pH 4.4 solution on intracellular reactive oxygen species and mitochondrial activity

An acidic solution (pH 4.4) increased the level of intracellular reactive oxygen species (ROS) detected by fluorescent ethidium (Fig. 3A). The NADPH oxidase (NOX) inhibitors DPI (10 μM) and gp91ds-tat (1 μM) did not decrease the level of pH 4.4-induced intracellular ROS (Fig. 3B). However, ROS scavengers (tiron, 1 mM and NAC, 5 mM) and mitochondrial inhibitors (rotenone, 5 μM and 5-HD, 100 μM) did inhibit pH 4.4-induced intracellular ROS. Furthermore, an acidic solution (pH 4.4) increased intracellular rhodamine 123 intensity, reflecting depolarization of the mitochondrial membrane potential (Fig. 3C). Mitochondrial inhibitors (rotenone, 5 μM and 5-HD, 100 μM) inhibited the increase in Rh-123 intensity produced by the pH 4.4 solution (Fig. 3D). The Cl^- channel inhibitor DIDS (100 μM) also inhibited pH 4.4

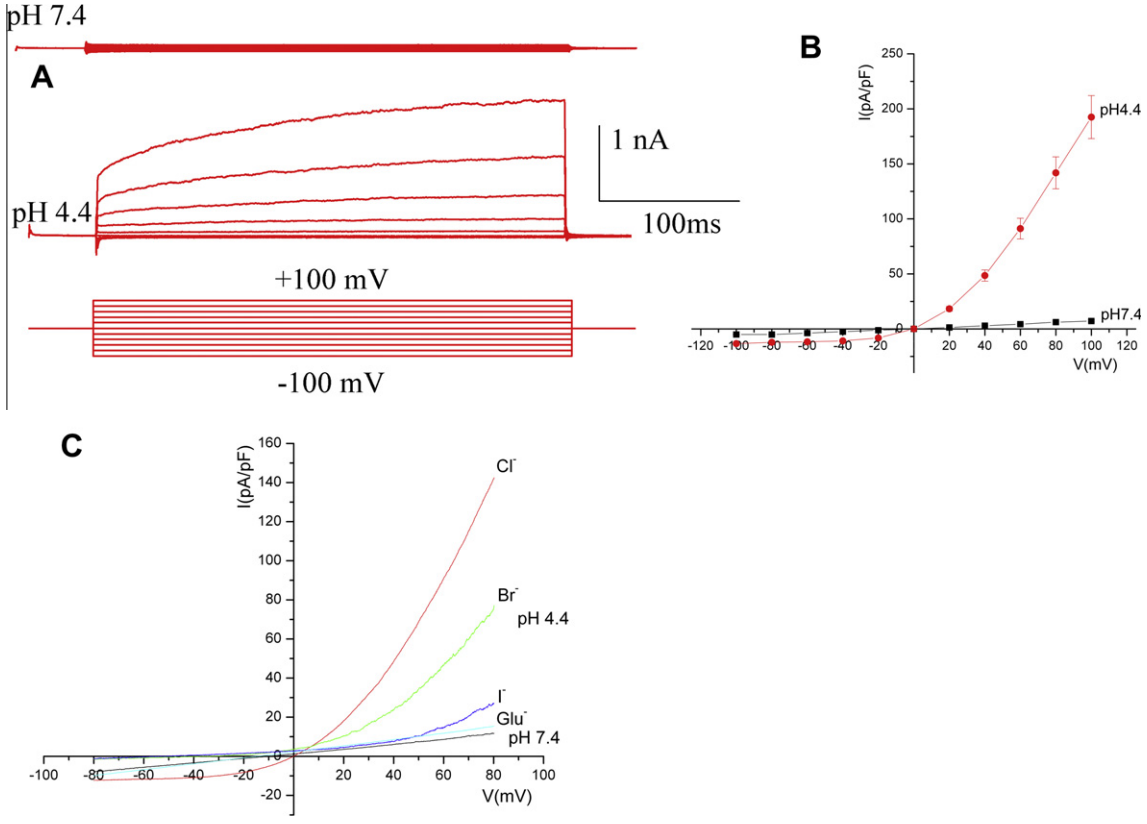

Fig. 2. Characteristics of acidic pH-induced currents (n = 10–12). (A) A representative current recording using a depolarizing step voltage protocol from −100 to +100 mV in 20 mV increments. At the depolarizing potentials, the current exhibited time-dependent facilitation and inactivation. (B) Mean current–voltage relationships also showed that the outward current exhibited a clear outward rectification with a reversal potential of 0 mV. (C) The relative anion permeability of this channel followed the sequence $Cl^- > Br^- > I^-$ > gluconate.

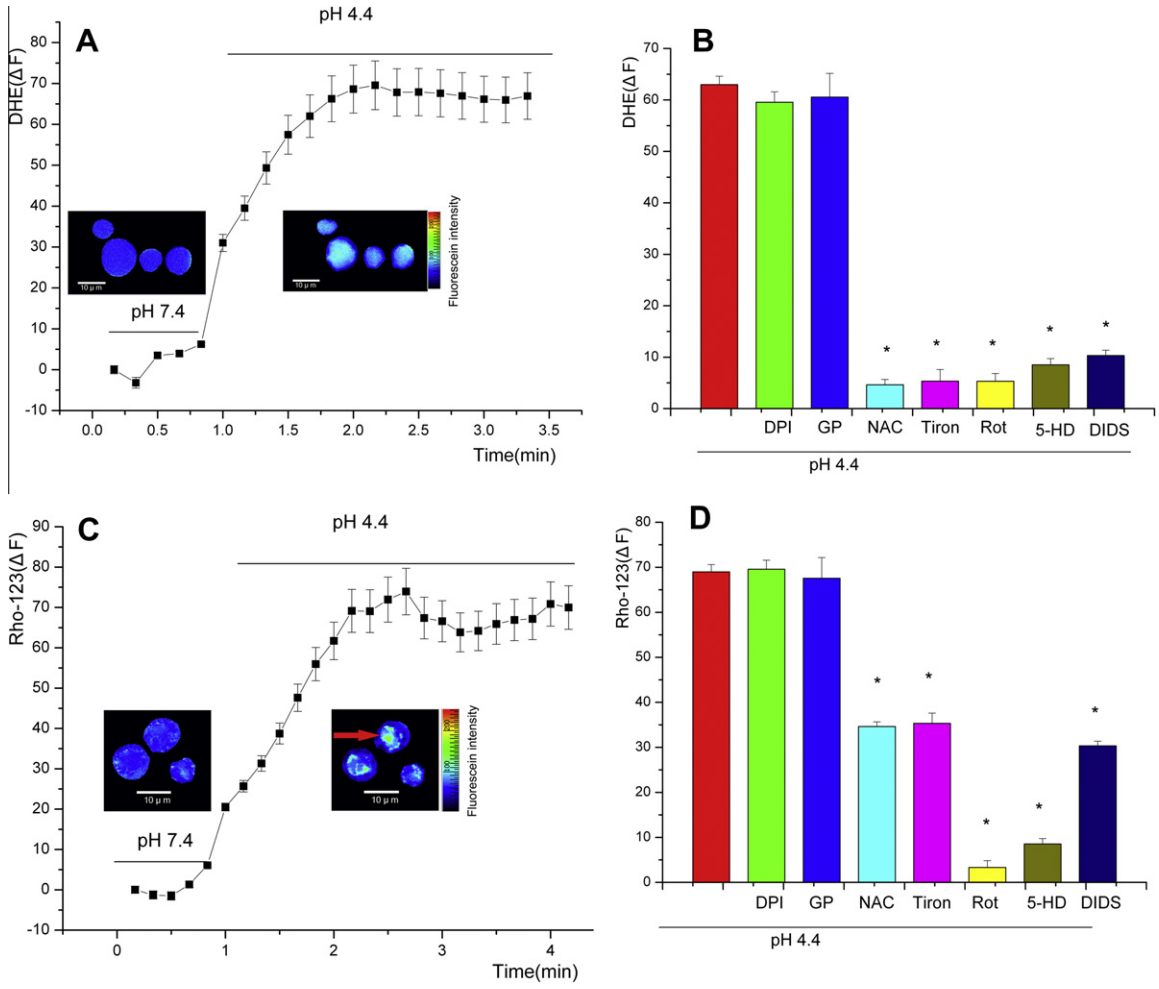

Fig. 3. Effect of a pH 4.4 solution on the level of intracellular reactive oxygen species and mitochondrial activity ($n = 50$). (A) A pH 4.4 (acidic) solution increased the level of intracellular reactive oxygen species (ROS) detected based on the fluorescence of ethidium. (B) The NADPH oxidase (NOX) inhibitors DPI (10 μM) and gp91ds-tat (1 μM) did not decrease pH 4.4-induced levels of intracellular ROS; ROS scavengers (tiron, 1 mM and NAC, 5 mM) and mitochondrial inhibitors (rotenone, 5 μM and 5-HD, 100 μM) did inhibit pH 4.4-induced intracellular ROS. (C) pH 4.4 solution also increased the intensity of Rh-123, reflecting depolarization of the mitochondrial membrane potential. (D) Mitochondrial inhibitors (rotenone, 5 μM and 5-HD, 100 μM) inhibited the increase in Rh-123 intensity induced by the pH 4.4 solution. DPI, diphenylene iodium; GP, gp91ds-tat; NAC, N-acetyl-L-cysteine; 5-HD, 5-hydroxydecanoate; Rh-123, rhodamine 123. $^*P < 0.01$, compared with data obtained at pH 4.4.

solution-induced intracellular ROS and mitochondrial membrane potential depolarization.

3.4. The effect of various intracellular ROS inhibitors on the current induced by pH 4.4 acidic solutions

The NADPH oxidase (NOX) inhibitors DPI (10 μM) and gp91ds-tat (1 μM) did not decrease the maximal currents induced by pH 4.4 acidic solution (Fig. 4A). However, ROS scavengers (tiron, 1 mM and NAC, 5 mM) and mitochondrial inhibitors (rotenone, 5 μM and 5-HD, 100 μM) did inhibit pH 4.4-induced currents (Fig. 4B and C). Representative current recordings confirmed these results (Fig. 4D).

4. Discussion

In the present study, we found that an extracellular acidic solution induced an outward current in human THP-1 cells. The current exhibited a clear outward rectification and pH dependence with a reversal potential of 0 mV. In THP-1 cells, this current was blocked using the Cl^- channel blocker DIDS. We also found that the relative anion permeability of this channel in THP-1 cells followed the sequence $Cl^- > Br^- > I^- >$ gluconate. Moreover, CsCl was used to inhibit potassium channel currents (the most widespread outward currents), and the sole anion in all of the tested solutions was Cl^-. Therefore, it could be concluded that this outward current was a Cl^- current, which was termed the extracellular acidic pH-activated, outward-rectifying chloride channel ($I_{Cl,acid}$).

The characteristics of $I_{Cl,acid}$ in THP-1 cells included outward rectification, time-dependent facilitation and inactivation during positive potentials. These properties are similar to those of $I_{Cl,acid}$ in other cells [4–7]. However, the current density activated at pH 4.4 in THP-1 cells was significantly greater than that of HUVECs (149.0 ± 5.9 vs. 29.87 ± 1.67 pA/pF) [6], and was similar to that of human monocytes (126.3 ± 31.7 pA/pF) [7]. The characteristics of $I_{Cl,acid}$ were also studied in cardiac myocytes [5]. Although some features are the same between these studies, the threshold pH, EC_{50} and current density are different. This may result from differences in the molecular structure of the forms of $I_{Cl,acid}$.

Because acidic microenvironments exert proinflammatory effects on immune cells [12,13], $I_{Cl,acid}$ may play a key role in the regulation of immune cell function during acidosis. However, the regulation of $I_{Cl,acid}$ remains largely unexplored. Most previous studies have found that the chloride transport inhibitor (DIDS) inhibits $I_{Cl,acid}$. Recently, Shi et al. found that $I_{Cl,acid}$ could be

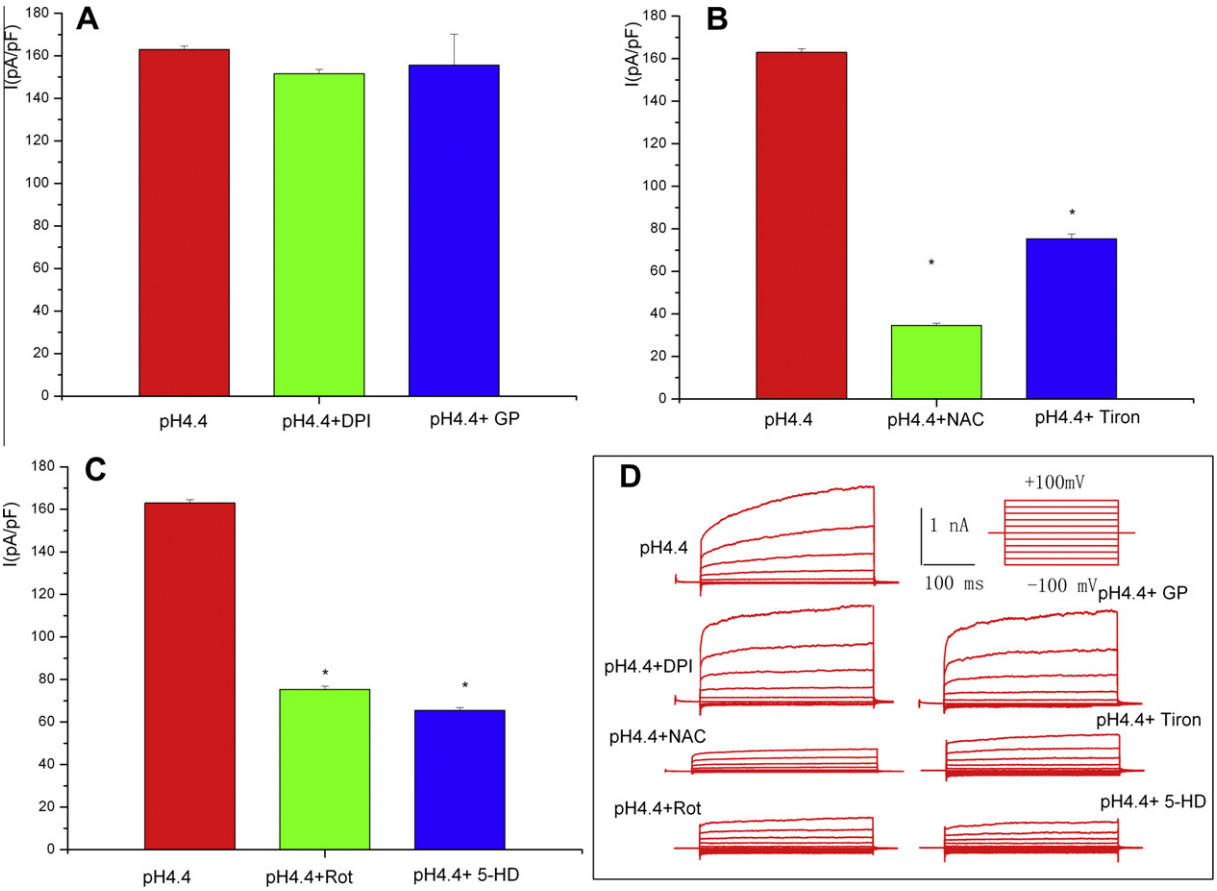

Fig. 4. The effect of various intracellular ROS inhibitors on the current induced by pH 4.4 (acidic) solution (n = 10–12). (A) The NADPH oxidase (NOX) inhibitors DPI (10 μM) and gp91ds-tat (1 μM) did not decrease the maximal currents induced by pH 4.4 acidic solution. (B) ROS scavengers (tiron, 1 mM and NAC, 5 mM) did inhibit the pH 4.4-induced currents. (C) Mitochondrial inhibitors (rotenone, 5 μM and 5-HD, 100 μM) also inhibited the pH 4.4-induced currents. (D) Representative current recordings using a depolarizing step voltage protocol from −100 to +100 mV in 20 mV increments. DPI, diphenylene iodium; GP, gp91ds-tat; NAC, N-acetyl-L-cysteine; 5-HD, 5-hydroxydecanoate; Rh-123, rhodamine 123. $^{*}P < 0.01$, compared with data obtained at pH 4.4.

inhibited by simvastatin [7]. In the present study, we found that the NADPH oxidase (NOX) inhibitors (DPI and gp91ds-tat) did not decrease the maximal currents induced by pH 4.4 acidic solution. However, ROS scavengers (tiron and NAC) and mitochondrial inhibitors (rotenone and 5-HD) did inhibit pH 4.4-induced currents. These results suggest that $I_{Cl,acid}$ may be regulated by intracellular ROS, which mainly originate from mitochondria. An increasing number of studies have shown that ROS activates many ion channels [14,15] and that antioxidants inhibit ion channels [16]. Therefore, the inhibitory effect of antioxidants on ion channel currents is seen in many studies, consistent with our results. The mechanism behind this phenomenon may be that ROS can oxidize key residues in ion channel proteins, thereby altering the gating properties of the ion channels [17,18].

Furthermore, fluorescence imaging of intracellular ROS levels and mitochondrial activity in THP-1 cells confirmed these findings in patch clamp experiments. An acidic solution (pH 4.4) increased the levels of intracellular ROS and mitochondrial activity. NADPH oxidase inhibitors did not decrease pH 4.4-induced intracellular ROS. However, ROS scavengers and mitochondrial inhibitors did inhibit pH 4.4-induced intracellular ROS. The Cl^- channel inhibitor DIDS also inhibited pH 4.4-induced intracellular ROS and depolarization of the mitochondrial membrane potential. In other studies, extracellular acidic pH was also associated with increased levels of intracellular ROS [8,9]. Therefore, $I_{Cl,acid}$ might represent a new target for regulating intracellular ROS in acidic environments.

In summary, our findingsreveal that an extracellular, acidic pH - activated, outward-rectifying chloride channel ($I_{Cl,acid}$) exists in human THP-1 cells. In addition, $I_{Cl,acid}$ is regulated by intracellular ROS levels, which mainly originate from mitochondria. Because an acidic environment has been demonstrated to exert proinflammatory effects on monocytes, $I_{Cl,acid}$ may be a useful new target for the regulation of monocyte function under acidic conditions. However, the molecular structure of $I_{Cl,acid}$ remains unclear; additional studies should be conducted to further elucidate the molecular mechanism of the effect of intracellular ROS on $I_{Cl,acid}$.

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