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## Regulation of endogenous and heterologous Ca<sup>2+</sup> release-activated Ca<sup>2+</sup> currents by pH\*

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### Abstract

Deviations from physiological pH (~ pH 7.2) as well as altered Ca<sup>2+</sup> signaling play important roles in immune disease and cancer. One of the most ubiquitous pathways for cellular Ca<sup>2+</sup> influx is the store-operated Ca<sup>2+</sup> entry (SOCE) or Ca<sup>2+</sup> release-activated Ca<sup>2+</sup> current (I<sub>CRAC</sub>), which is activated upon depletion of intracellular Ca<sup>2+</sup> stores. We here show that extracellular and intracellular changes in pH regulate both endogenous I<sub>CRAC</sub> in Jurkat T lymphocytes and RBL2H3 cells, and heterologous I<sub>CRAC</sub> in HEK293 cells expressing the molecular components STIM1/2 and Orai1/2/3 (CRACM1/2/3). We find that external acidification suppresses, and alkalization facilitates IP<sub>3</sub>-induced I<sub>CRAC</sub>. In the absence of IP<sub>3</sub>, external alkalization did not elicit endogenous I<sub>CRAC</sub> but was able to activate heterologous I<sub>CRAC</sub> in HEK293 cells expressing Orai1/2/3 and STIM1 or STIM2. Similarly, internal acidification reduced IP<sub>3</sub>-induced activation of endogenous and heterologous I<sub>CRAC</sub>, while alkalization accelerated its activation kinetics without affecting overall current amplitudes. Mutation of two aspartate residues to uncharged alanine amino acids (D110/112A) in the first extracellular loop of Orai1 significantly attenuated both the inhibition of I<sub>CRAC</sub> by external acidic pH as well as its facilitation by alkaline conditions. We conclude that intra- and extracellular pH differentially regulates I<sub>CRAC</sub>. While intracellular pH might affect aggregation and/or binding of STIM to Orai, external pH seems to modulate I<sub>CRAC</sub> through its channel pore, which in Orai1 is partially mediated by residues D110 and D112.

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## Keywords

STIM; Orai; HEK293; Jurkat cells; RBL; whole-cell patch clamp;  $I_{CRAC}$

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## 1. INTRODUCTION

Both,  $Ca^{2+}$  signaling and pH are altered in different pathophysiological circumstances such as immune disease and cancer [1-5]. In this context, the pH dependence of  $Ca^{2+}$ -permeable ion channels is of particular interest since they are the main provider of the key intracellular second messenger  $Ca^{2+}$ . All  $Ca^{2+}$  channels examined so far seem to share the same basic pH responsiveness: extracellular acidification reduces and alkalization increases the amplitude of  $Ca^{2+}$  inward currents [6-8]. For the L-type voltage-gated  $Ca^{2+}$  channel it is suggested that the molecular basis for the effect of extracellular pH changes is a protonation site that lies within the pore and which is formed by a combination of conserved glutamate residues [9]. However, for most pH-dependent effects on  $Ca^{2+}$  channels, the underlying mechanisms are not well understood and might differ from each other.

One universally present  $Ca^{2+}$  influx mechanism in mammalian cells is the store-operated  $Ca^{2+}$  entry (SOCE) [10]. STIM1 and Orai1 (CRACM1) were identified as main molecular components of SOCE and the underlying  $Ca^{2+}$  release-activated  $Ca^{2+}$  current ( $I_{CRAC}$ ) [11-16]. Upon store depletion, the endoplasmic reticulum  $Ca^{2+}$  sensor STIM1 aggregates and translocates into junctional structures close to the plasma membrane where it binds and activates Orai1,  $Ca^{2+}$ -selective ion channels, that have been described as either a tetramer or a hexamer [17-25]. Besides STIM1 and Orai1, the isoforms STIM2, Orai2 and Orai3 are ubiquitously expressed and can form functional channels with distinct biophysical and pharmacological properties [26-33].

Various  $Ca^{2+}$  imaging studies have demonstrated that an acidic pH can contribute to lowered SOCE signals in several cell types [34-39]. All these studies show that extracellular acidification decreases and alkalization increases SOCE. Patch-clamp studies of  $I_{CRAC}$  corroborate the  $Ca^{2+}$  imaging results.  $I_{CRAC}$  in human monocyte-derived macrophages shows an extracellular pH-dependent change of current amplitude with a  $pK_a$  of 8.2 [40]. Two studies in Jurkat T lymphocytes suggest that extracellular pH modifies the mitochondrial control of SOCE in Jurkat cells [41,42]. Others predict a similar mechanism as assumed for L-type  $Ca^{2+}$  channels via the protonation of negatively charged glutamate residues close to the channel pore [43,44]. The glutamate residue E106, which is known to contribute to the selectivity filter of multimeric Orai1 channels [45-47], appears to be involved in the pH dependence of heterologously expressed STIM1/Orai1 CRAC channels [48]. Mutation of E106 to aspartic acid in Orai1 (E106D) resulted in a reverse pH dependence compared to Orai1 wild type: while Orai1 is normally blocked upon external acidification, Orai (E106D) exhibits an increase in inward current at pH 5.1. Hence, protonation of the E106 residue might change the pore size and also  $Ca^{2+}$  binding inside the pore, resulting in a block of Orai1.

So far, most of the information regarding pH effects on SOCE or  $I_{CRAC}$  has been gathered by  $Ca^{2+}$  imaging experiments from diverse cell types or from experiments with heterologous

co-expression of Orai1 and STIM1. Here, we investigate and compare the effects of external and internal pH on the diverse combinations of heterologous STIM/Orai mediated  $I_{CRAC}$  in HEK293 cells, and endogenous  $I_{CRAC}$  in the RBL2H3 mast cell line and Jurkat T lymphocytes.

## 2. RESULTS

### 2.1 Extracellular pH modulates endogenous $I_{CRAC}$

To study the effects of extracellular pH on endogenous  $I_{CRAC}$  in RBL2H3 cells and Jurkat T lymphocytes,  $I_{CRAC}$  was first activated via  $IP_3$  in the patch pipette at an external pH of 7.2 and then bath solutions of different pH were applied after  $I_{CRAC}$  had fully developed. Figures 1A and B show, that for both cell types the amplitude of  $I_{CRAC}$ , measured at -80 mV, increased upon external alkalization (Fig. 1A) and decreased upon external acidification (Fig. 1B). For quantitative analysis of the pH-dependent current changes, all single cell currents were normalized to cell size (pA/pF) and to the amplitude of the inward current right before pH change (here  $I/I_{120s}$ ), and then averaged (see Fig. 1C for RBL2H3 cells; upper traces show the outward currents). Finally, the relative changes of the inward current ( $I_{CRAC}$ ;  $I/I_{120}$ ) amplitudes were plotted versus the external pH (Fig. 1D). A double dose-response equation (see Material and Methods) best represented the dose (pH) response ( $I/I_{120}$ ) relationships, and the corresponding fits revealed a  $pK_a$  (for acidification) of 6.8 and  $pK_b$  (for alkalization) of 8.0 for Jurkat T lymphocytes, and a  $pK_a$  of 6.7 and  $pK_b$  of 7.8 for RBL2H3 cells (Fig. 1D). Changing to external pH 6.0 inhibited  $I_{CRAC}$  by more than 80% in both cell types, whereas pH 8.4 increased inward currents in RBL2H3 cells about 24% and in Jurkat T lymphocytes about 80% (Fig. 1D). Outward currents were not significantly affected except for a small current increase of less than 0.5 pA/pF during alkalization (see Figs. 1C, E and F). Figures 1E and F show the inwardly rectifying I/V relationships typical for the  $Ca^{2+}$ -selective  $I_{CRAC}$  at different external pH from RBL2H3 cells and Jurkat T lymphocytes, respectively. However, in Jurkat T lymphocytes the current at pH 8.4 exhibits a small outward component which might be due to an alternative channel more prevalent in Jurkat T lymphocytes than in RBL2H3 cells.

### 2.2 pH effects on heterologously expressed STIM/Orai-mediated $I_{CRAC}$ in HEK293 cells

In order to investigate whether heterologous expression of STIM1/2 and Orai1-3 molecules exhibit the same pH dependency as endogenous  $I_{CRAC}$  in RBL2H3 and Jurkat T lymphocytes, we transfected Orai1, Orai2 and Orai3 into HEK293 cells stably expressing STIM1 or STIM2. Cells were used 24 to 48 hours after transfection. In analogy to the experiments shown in Fig. 1, bath solutions of pH 6.0, 6.6, 7.8, 8.4 and 9.0 were applied after full development of  $IP_3$ -induced  $I_{CRAC}$  at 120 s. Fig. 2A shows the pH-dependent changes of  $I_{CRAC}$  amplitudes in STIM1/Orai1-expressing HEK293 cells (S1O1), Fig. 2B for STIM1/Orai2 (S1O2), and Fig. 2C for STIM1/Orai3 (S1O3). Plotting the relative pH-dependent current changes ( $I/I_{120s}$ ) versus the corresponding external pH, revealed similar acidification-dependent decreases of  $I_{CRAC}$  in S1O1-, S1O2- and S1O3-expressing HEK293 cells (Fig. 2D) as those observed for endogenous  $I_{CRAC}$  in RBL2H3 cells and Jurkat T lymphocytes (see Fig. 1D). However,  $I_{CRAC}$  in S1O1-, S1O2- and S1O3-expressing HEK293 cells was more amplified upon alkalization (up to 300%, Fig. 2D) than endogenous

$I_{CRAC}$  in RBL2H3 and Jurkat T lymphocytes (up to ~120% - 180%) (Fig. 1D). The same double dose-response equation as used in Fig. 1D was used to fit the dose-response relationships in Fig. 2D. The  $pK_a$  (acidification) and  $pK_b$  (alkalization) for S1O1- and S1O2-expressing HEK293 cells (both  $pK_a$  6.8,  $pK_b$  7.9) were comparable to  $pK_a$  and  $pK_b$  for endogenous  $I_{CRAC}$  in RBL2H3 cells and Jurkat T lymphocytes (see above). S1O3-expressing HEK293 cells ( $pK_a$  6.7,  $pK_b$  8.4) revealed a slightly higher  $pK_b$ . Fig. 2F shows the typical inwardly rectifying I/V relationships for  $I_{CRAC}$  in S1O1-, S1O2-, and S1O3-expressing HEK293 cells at external pH 7.2 (upper curves) and 8.4 (lower curves). As seen in RBL2H3 and Jurkat T lymphocytes, small outward currents appeared during external alkalization (see I/Vs in Fig. 2F).

Under physiological conditions  $I_{CRAC}$  is a highly selective  $Ca^{2+}$  current. To exclude pH-dependent changes in ion selectivity we replaced the external monovalent cation  $Na^+$  by the ion channel-impermeable cation tetraethylammonium ( $TEA^+$ ).  $Na^+$ -free conditions did not prevent the massive alkalization-induced current increase (Fig. 2E). In addition, no further outward current appeared after external alkalization (see Fig. 2E upper traces and I/Vs in Fig. 2F). This suggests that the alkalization-mediated current increase in STIM1/Orai1-expressing cells is due to an increased  $Ca^{2+}$  influx and not to a change in ion selectivity.

Similar to STIM1, STIM2 also mediates large  $I_{CRAC}$  currents. When co-expressed with Orai1-3 STIM2 mediates a store-dependent (first phase – fast, small amplitude) and store-independent (second phase – slow, high amplitude) mode of CRAC channel activation [28]. We transiently expressed Orai1 and Orai3 into HEK293 cells stably expressing STIM2 to see whether STIM2-mediated  $I_{CRAC}$  shows the same alkalization-induced increase as seen for STIM1. As previously shown,  $IP_3$ -induced  $I_{CRAC}$  in STIM2/Orai1 (S2O1)-expressing cells produced a significantly larger second-phase current than STIM2/Orai3 (S2O3)-expressing HEK293 cells (Figs. 2G and H; filled circles). External alkalization resulted in an increase of current in both S2O1- and S2O3-expressing cells (Figs. 2G and H; open circles). Fig. 2I illustrates the I/V relationships of  $IP_3$ -induced  $I_{CRAC}$  in S2O1- and S2O3-expressing HEK293 cells at external pH 7.2 and 8.4. As already observed in  $IP_3$ -induced STIM1-mediated  $I_{CRAC}$ , outward currents were largely unaffected by external alkalization. Figures 2G and 2H show that the alkalization-induced current potentiation in S2O1- and S2O3-expressing HEK293 cells increased along with the rising secondary phase of  $I_{CRAC}$ , and, after applying pH 7.2, the current amplitude declined quickly to levels of control cells (without pH change). This suggests that the alkalization-dependent potentiation of S2O1-mediated  $I_{CRAC}$  seems to somehow boost activated open channels, rather than recruiting further closed channels.

Wild type HEK293 cells (HEK wt) and HEK293 cells just expressing Orai1-3, all reveal  $IP_3$ -induced  $I_{CRAC}$  currents at a basal pH of 7.2 of maximally 1 pA/pF (see supplementary Fig. 1). Alkalization (pH 8.4) increased endogenous  $IP_3$ -induced  $I_{CRAC}$  in HEK wild type cells about 65%, in Orai1- and Orai2-expressing HEK293 cells about 130%, and in Orai3-expressing cells about 320% (Fig. S1A, I/Vs in Fig. S1B and S1C). The relative increase of current is comparable to the alkalization-mediated increase of  $IP_3$ -induced  $I_{CRAC}$  in STIM/Orai-expressing HEK293 cells, but the absolute current amplitudes are substantially smaller. This suggests, that after store-depletion only Orai channels, which are stimulated by STIM

are boosted by extracellular alkalization, and additional Orai channels do not open upon extracellular alkalization without contribution of STIM, which is limited in Orai1-3-expressing HEK293 cells.

### 2.3 Induction of heterologous $I_{CRAC}$ upon extracellular alkalization

The previous experiments assessed the effect of extracellular pH on  $I_{CRAC}$  that was already activated by  $IP_3$ -induced store depletion. In the next set of experiments, no  $IP_3$  was added to the intracellular solution of the patch pipette. Instead, the intracellular  $Ca^{2+}$  concentration was buffered to 150 nM to prevent passive store depletion and thus activation of  $I_{CRAC}$ . Under these conditions, RBL2H3, Jurkat T lymphocytes, HEK293 wt cells or HEK293 cells expressing Orai1, Orai2 or Orai3 do not develop  $I_{CRAC}$ . Extracellular alkalization (pH 8.4) evoked a small linear current (Figs. 3A and B; I/Vs are shown in 3D and E), which was largest in Orai1- and Orai2-expressing HEK293 and RBL2H3 cells, but still less than 1.5 pA/pF. Only Orai3-expressing HEK293 cells revealed a small current with an inward rectifying I/V-relationship, but this current was also much smaller than in the presence of pH 8.4 after store depletion (Fig. S1). These experiments support our previous findings (see above) that Orai molecules forming the CRAC channels are not directly activated by extracellular alkalization. However, the additional expression of STIM1/2 in Orai1-3-expressing HEK293 cells changed the response to alkalization: without apparent store-depletion, extracellular alkalization induced large  $I_{CRAC}$  in S1O1-, S1O2-, S1O3- and S2O1-expressing HEK293 cells (Figs. 3C and F; Fig. S2A-E). The  $EC_{50}$  for alkalization induced  $I_{CRAC}$  in S1O1-expressing HEK293 cells is 7.8 (Fig. 3I).

In analogy to the electrophysiological results, imaging experiments revealed no  $Ca^{2+}$  influx upon application of pH 8.4 onto intact Fura-2 AM loaded RBL2H3 and only a weak intracellular  $Ca^{2+}$  increase in HEK wt cells (Fig. 3G). In contrast, pH 8.4 induced a significant  $Ca^{2+}$  increase in Jurkat T lymphocytes, which was prevented in the absence of external  $Ca^{2+}$  (Fig. 3H). This demonstrates that the application of external pH 8.4 did not result in store depletion, whereas control application of thapsigargin did (Fig. 3H). Consistent with the patch-clamp results and activation of inward currents, S1O2-, S1O3- and S2O1-expressing HEK293 cells also revealed an increase in intracellular  $Ca^{2+}$  upon extracellular alkalization, which depended on extracellular  $Ca^{2+}$  (Fig. S2F).

### 2.4 Intracellular effects of pH on endogenous and STIM1/Orai1-mediated $I_{CRAC}$

We next studied the effect of intracellular pH on  $IP_3$ -induced  $I_{CRAC}$  in S1O1-expressing HEK293 cells (Figs. 4A and C), RBL2H3 cells (Figs. 4B and C) and Jurkat T lymphocytes (Fig. 4C). Intracellular acidification inhibited  $IP_3$ -induced  $I_{CRAC}$ , whereas intracellular alkalization did not significantly change its amplitude (Figs. 4A and C). Upper traces in Fig. 4A and B show the outward currents. The dose (pH) - response (I) relationships of intracellular pH on  $I_{CRAC}$  are summarized in Fig. 4C. The mean currents at different intracellular pH were normalized to the maximum current for each cell type. Sigmoidal fits revealed a pK of 6.7 for S1O1-expressing HEK293 cells, 6.4 for RBL2H3 cells and 6.2 for Jurkat T lymphocytes. At an intracellular pH of 6.0 no significant  $I_{CRAC}$  could be detected in any cell type. To see whether pH 6.0 inhibited  $I_{CRAC}$  due to a pH-dependent inhibition of store depletion via  $IP_3$  we applied 2  $\mu$ M ionomycin to deplete intracellular  $Ca^{2+}$  stores

independently of IP<sub>3</sub> (Fig. 4D). The application of ionomycin did not activate any additional current in S1O1-expressing HEK293 cells, RBL2H3 cells and Jurkat T lymphocytes treated with intracellular IP<sub>3</sub> at an intracellular pH of 6.0 (Fig. 4D).

Since intracellular alkalization did not change the amplitude of IP<sub>3</sub>-induced I<sub>CRAC</sub> we further studied whether intracellular alkalization had any effect on its extracellular alkalization-induced potentiation. Fig. 4E shows, that the potentiation of IP<sub>3</sub>-induced I<sub>CRAC</sub> by an external application of pH 8.4 at an internal pH of 7.2 and 8.4 was not different in S1O1-expressing HEK293 cells. Neither was the relative acidification-induced (pH 6.0) inhibition of IP<sub>3</sub>-induced I<sub>CRAC</sub> in the presence of internal pH 7.2 and 9.0 (Fig. 4F). However, the IP<sub>3</sub>-induced I<sub>CRAC</sub> in S1O1-expressing HEK293 cells and RBL2H3 cells developed faster at alkalized intracellular conditions (see also Figs. 4A, B, E and F).

### 2.5 Mutations of D110/112A in Orai1 changes its pH profile

Close to the glutamate residue in position 106 (E106), which has already been shown to mediate some pH dependence [48], the first extracellular loop of Orai1 reveals two additional negatively charged residues: aspartate residues D110 and D112. An Orai1 construct in which these two aspartates were mutated to uncharged alanine residues (D110/112A) demonstrated that these residues contribute to the ion selectivity and to the anomalous mole-fraction behavior of Orai1 channels [46]. We tested for the pH dependence of I<sub>CRAC</sub> via Orai1<sub>D110/112A</sub> compared to Orai1<sub>wt</sub>. We applied pH 6.0 or pH 8.4 after IP<sub>3</sub>-activated I<sub>CRAC</sub> had developed in HEK293 cells stably expressing STIM1 and either Orai1<sub>wt</sub> or Orai1<sub>D110/112A</sub>. Currents were normalized to IP<sub>3</sub>-induced currents at t=120 s and plotted vs. time (Fig. 5A, I/Vs are shown in 5B). Fig. 5C demonstrates that the alkalization-induced amplification of I<sub>CRAC</sub> upon application of pH 8.4 was significantly smaller in Orai1<sub>D110/112A</sub> compared to Orai1<sub>wt</sub> (Fig. 5C). In addition, the bar diagram in Fig. 5D demonstrates that the residual current upon pH-induced block (pH 6.0) was significantly larger in Orai1<sub>D110/112A</sub> compared to Orai1<sub>wt</sub>.

## 3. DISCUSSION

In the present study we assessed extra- and intracellular pH changes on the development and amplitude of the Ca<sup>2+</sup> release-activated Ca<sup>2+</sup> current (I<sub>CRAC</sub>) in HEK293 cells heterologously expressing STIM and Orai proteins and endogenous I<sub>CRAC</sub> in RBL2H3 cells as well as Jurkat T lymphocytes.

In agreement with previous studies investigating extracellular pH effects on store-operated Ca<sup>2+</sup> entry (SOCE) via fluorescent Ca<sup>2+</sup> imaging [34-39] and/or I<sub>CRAC</sub> via electrophysiological techniques [40] we found that extracellular acidification decreases and alkalization increases I<sub>CRAC</sub> in endogenous cells as well as in STIM/Orai co-overexpressing HEK cells (S1O1, S1O2, S1O3, S2O1, S2O3). Since the pH/current relationships reveal very similar pKs for acidification-induced inhibition (6.7-6.8) and pKs for alkalization-induced potentiation (7.8-8.4) in both endogenous and heterologously expressed I<sub>CRAC</sub>, we assume that the extracellular pH dependency is a general feature of I<sub>CRAC</sub>. In RBL2H3 cells and Jurkat T lymphocytes the differences in pK values might result from different expression levels of STIM and Orai homologues.

Recently, the glutamate residue E106 in Orai1 has been identified to contribute to the extracellular pH dependence of  $\text{Ca}^{2+}$  release-activated  $\text{Ca}^{2+}$  (CRAC) channels [48]. Here, we demonstrate that two aspartate residues in the first extracellular loop of Orai1, close to E106, also contribute to the pH-induced changes of  $I_{\text{CRAC}}$ . The lower degree of amplification of Orai1<sub>D110/112A</sub> could be explained by the absence of negative charges, resulting in a missing energy-well for the  $\text{Ca}^{2+}$  coordination of the first loop close to the ion channel pore. In conclusion a protonation of D110/112 could decrease current size at low pH. The less efficient block of Orai1<sub>D110/112A</sub> at low pH is possibly due to changed steric arrangement of the uncharged alanine residues compared to the protonated aspartate residues probably increasing Orai channel conductance.

Extracellular alkalization of Orai1-3 in the absence of STIM did not result in a substantial increase of CRAC currents. This suggests that the potentiation by extracellular pH only affects Orai channels that interact with STIM. However,  $I_{\text{CRAC}}$  and  $\text{Ca}^{2+}$  influx in HEK293 cells co-overexpressing different STIM and Orai combinations as well as  $\text{Ca}^{2+}$  influx in Jurkat T lymphocytes can be activated by extracellular alkalization without apparent store-depletion. Taken together, our data suggest that extracellular alkalization can activate  $I_{\text{CRAC}}$  in the STIM/Orai overexpression system, but apparently no endogenous  $I_{\text{CRAC}}$ . Since no alkalization-mediated endogenous  $I_{\text{CRAC}}$  was detectable in HEK wt, RBL and Jurkat T lymphocytes, it remains unclear whether the alkalization-mediated  $\text{Ca}^{2+}$  influx in Jurkat T lymphocytes depends in endogenous STIM/Orai channels, or is mediated via other  $\text{Ca}^{2+}$  influx pathways in these cells.

Intracellular pH also affects  $I_{\text{CRAC}}$ . Our results show that intracellular acidification inhibits both endogenous and heterologous  $I_{\text{CRAC}}$ , while intracellular alkalization did not alter the current amplitude. The internal pH-dependent inactivation of  $I_{\text{CRAC}}$  in RBL cells correlates with a pH-dependent inactivation of iPLA<sub>2</sub> $\beta$  [49], which is already shown to be a modulator of  $I_{\text{CRAC}}$  [50]. However, in human neuroblastoma cells, intracellular changes in pH left carbachol-induced  $\text{Ca}^{2+}$  entry unaffected [35]. Since we induced store-depletion by  $\text{IP}_3$  already in the presence of the diverse pH concentrations in the patch pipette, the decrease in  $I_{\text{CRAC}}$  activation at a lower intracellular pH might be due to an acidic-pH-dependent inhibition of  $\text{IP}_3$  binding to its receptor in the membrane of the endoplasmic reticulum, resulting in a slower  $\text{Ca}^{2+}$  store depletion and thus  $I_{\text{CRAC}}$  activation [51]. However, a further  $\text{IP}_3$  receptor-independent induction of store depletion by the  $\text{Ca}^{2+}$  ionophore ionomycin did not result in the CRAC currents at an intracellular pH of 6.0 either. These results imply that intracellular acidification directly blocks the STIM/Orai machinery. This concept is supported by two recent studies. Investigations of reduced SOCE in human alveolar smooth muscle cells upon hypoxia-induced intracellular acidification and  $I_{\text{CRAC}}$  in HEK293 cells heterologously expressing STIM1 and Orai1 suggest an uncoupling of STIM1 and Orai1 due the intracellular drop of pH, which might be an important mechanism to protect cells from  $\text{Ca}^{2+}$  overload under hypoxic stress [52]. In addition, experiments with STIM1 siRNA in pheochromocytoma cells demonstrate that changes in pH alter the ability of STIM1 to trigger SOCE either due to inhibition of conformational changes or decreased STIM1 translocation or aggregation [53].

Several publications discuss a reduced or increased store-operated  $\text{Ca}^{2+}$  entry along with cancer- E.g., lowered SOCE-conducted  $\text{Ca}^{2+}$  signaling can result in typical cancer hallmark functions such as enhanced proliferation, inability to induce apoptosis and a higher migration potential [54-56]. Tumor environment is often characterized by an acidic pH [57] and various chemical and technical approaches use acidic tumor environment to unfold their therapeutic potential [58-61]. Recent work demonstrated a role for STIM/Orai isoforms STIM2 and Orai3 in the pathophysiology of cancer [62-69]. Our results imply that low pH in tumor environment might contribute to low SOCE signaling.

In addition, the strong external pH-sensitivity might be of physiological significance in the activation of immune cells. Low extracellular pH can contribute significantly to the modulation of SOCE in cells at sites of inflammation. In the microenvironment of abscesses, the external pH can be as low as 5.5 [70] and acidic microenvironments may play a role in inhibiting immune function as suggested to be the case in cystic fibrosis [71]. The acidification-induced inhibition of  $I_{\text{CRAC}}$ , most probably one of the most important factors in immune cell activation, might well be the reason for the well-known diminished immune response at lowered extracellular pH. However, the reduction of  $\text{Ca}^{2+}$  influx in acidic environment may also be an intrinsic protective feedback mechanism in inflammatory cells.

## CONCLUSION

In cancer and immune reaction extracellular pH is lowered and  $\text{Ca}^{2+}$  signals are impaired. Endogenous and STIM/Orai mediated  $I_{\text{CRAC}}$  depend on intra- and extracellular pH and two aspartic acids in the pore of Orai1 contribute to the extracellular pH-dependence. Inhibition of  $\text{Ca}^{2+}$  influx via Orai channels upon extracellular acidification is a general feature of  $I_{\text{CRAC}}$  and lowered pH in tumors and immune response can thus contribute to  $\text{Ca}^{2+}$  dependent malfunctions.

## 4. MATERIAL AND METHODS

### 4.1 Cells

HEK293 wild type (wt) and stably expressing STIM1 or STIM2 cells [72] were cultured at 37°C with 5%  $\text{CO}_2$  in DMEM supplemented with 10% fetal bovine serum (FBS). Before experiments, STIM2-expressing cells were grown for several days or weeks in the absence of G418 (500  $\mu\text{g}/\text{ml}$ ; see [28]). Full length human Orai1, Orai2 and Orai3 were subcloned into pCAGGS-IRES-GFP for transient dicistronic expression with the green fluorescent protein (GFP) as described previously [27]. For electrophysiological experiments, Orai1, Orai2 and Orai3 proteins were over-expressed in HEK293 wt or stably expressing STIM1 or STIM2 cells using lipofectamine 2000 (Invitrogen). GFP-expressing cells were selected by fluorescence. Experiments were performed 24-48 hours after transfection.

Jurkat T lymphocytes and RBL2H3 cells were cultured at 37°C with 5%  $\text{CO}_2$  in RPMI-1640 medium and in DMEM medium, respectively, both supplemented with 10% FBS and 0.1% penicillin-streptomycin.



## 4.2 Electrophysiology

Patch pipettes, pulled from glass capillaries (inner diameter 1.5 mm, Kimble products) with a horizontal puller (Sutter instruments, Model P-97), were fire-polished, and had resistances between 2 and 4 M $\Omega$ . Patch clamp experiments were performed in the tight-seal whole-cell configuration at 21-25 °C. High-resolution current recordings were acquired and analyzed using the EPC-10 and the software PatchMaster (HEKA). Voltage ramps of 50 ms duration spanning a range of -100 to +100 mV (or -150 to +150 mV) were delivered from a holding potential of 0 mV at a rate of 0.5 Hz over a period of 120-600 s. All voltages were corrected for a liquid junction potential of 10 mV. Currents were filtered at 2.9 kHz and digitized at 100  $\mu$ s intervals. Capacitive currents were determined and corrected before each voltage ramp. Extracting the current amplitude at -80 mV and +80 mV from individual ramp current records assessed the low-resolution temporal development of inward and outward currents, respectively. All currents were normalized to the cell size and displayed as current densities (pA/pF). Standard external solutions contained (in mM): 120 NaCl, 2 MgCl<sub>2</sub>, 10 CaCl<sub>2</sub>, 10 TEA-Cl, 10 HEPES, 10 glucose, pH 7.2 with NaOH, 300 mOsm. In some experiments, NaCl was replaced equimolarly by tetraethylammonium-chloride (TEA-Cl). The external solution was set to different pH by adding NaOH or HCl and pressure-applied directly onto the cell of interest via a wide-tipped puffer pipette. If necessary, osmolarity was adjusted by adding sucrose. Standard internal solutions contained (in mM): 120 Cs-glutamate, 20 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid tetra cesium salt (Cs-BAPTA), 3 MgCl<sub>2</sub>, 10 HEPES, 0.02 IP<sub>3</sub>, pH 7.2 with CsOH, 300 mOsm. For some experiments IP<sub>3</sub> was omitted and [Ca<sup>2+</sup>]<sub>i</sub> was buffered to 150 nM using 20 mM Cs-BAPTA and 8 mM CaCl<sub>2</sub> (8.5 mM for pH 8.4) as calculated with WebMaxC, <http://www.stanford.edu/~cpatton/webmaxcS.htm>). The intracellular solution was set to different pH by adding CsOH or HCl. All chemicals were purchased from Sigma-Aldrich Co (St. Louis, MO, USA). For some experiments, 2  $\mu$ M ionomycin (Sigma) was applied extracellularly.

## 4.3 Fluorescence measurements

For Ca<sup>2+</sup> measurements, cells were loaded with fura-2 AM (Molecular Probes, Eugene, OR, USA; 1  $\mu$ M / 60 min / 37 °C) and kept in extracellular saline containing 10 mM CaCl<sub>2</sub>. External solution of pH 8.4 with or without 10 mM CaCl<sub>2</sub> or external solution (pH 7.4) without CaCl<sub>2</sub> but 2  $\mu$ M thapsigargin were applied onto the cell of interest via puffer pipette. Experiments were performed with a Zeiss Axiovert 200 fluorescence microscope equipped with a dual excitation fluorimetric imaging system (TILL-Photonics, Gräfelfingen, Germany), using a 40x Plan NeoFluar objective. Data acquisition and computation were controlled by X-Chart (HEKA). Cells were excited by wavelengths of 360 nm and 390 nm produced by a monochromator B (TILL-Photonics). The fluorescence emission was recorded with a photomultiplier tube (TILL-Photonics) using an optical 440 nm longpass filter. The signals were sampled at 5 Hz and computed into relative ratio units of the fluorescence intensity at the different wavelengths (360/390 nm). Results are given as the approximate [Ca<sup>2+</sup>]<sub>i</sub>, calculated from the 360/390 nm fluorescence ratios, using an *in vivo* Ca<sup>2+</sup> calibration performed in patch-clamp experiments with defined Ca<sup>2+</sup> concentrations

combined with 200  $\mu$ M fura-2 pentapotassium salt (Molecular Probes, Eugene, OR, USA) in the patch pipette.

#### 4.4 Statistics and Analysis

Double dose-response equations ( $y=y_{\text{max}1}/1+(pH/pK_a)^{n_1}+y_{\text{max}2}/1+(pH/pK_b)^{n_2}$ ,  $y=I/I_{120s}$ ,  $pK_a$  and  $n_1 = pK$  and Hill coefficient for external acidification, and  $pK_b$  and  $n_2 = pK$  and Hill coefficient for external alkalization) were used to represent the dose (external pH) - response ( $I/I_{120}$ ) relationships. Sigmoidal fits ( $y=y_{\text{max}}/1+(pH/pK)^n$ ,  $y=I/I_{\text{max}}$ ,  $pK$  and  $n =$  Hill coefficient) were used to represent the dose (internal pH) - response ( $I/I_{120}$ ) relationships. Where applicable, statistical errors of averaged data are given as means  $\pm$ SEM with  $n$  determinations.

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

### Acknowledgments

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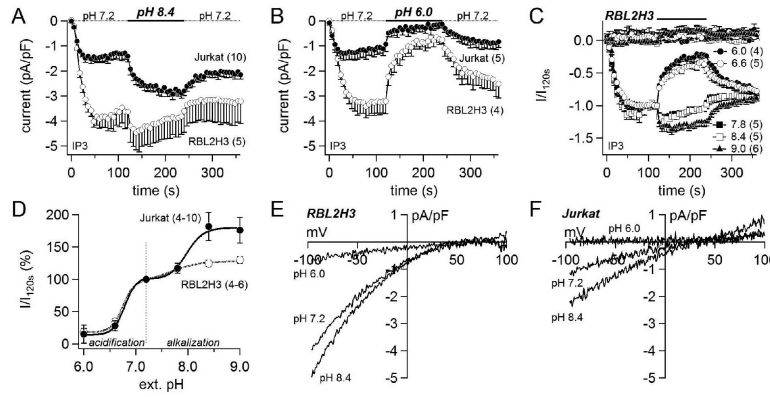
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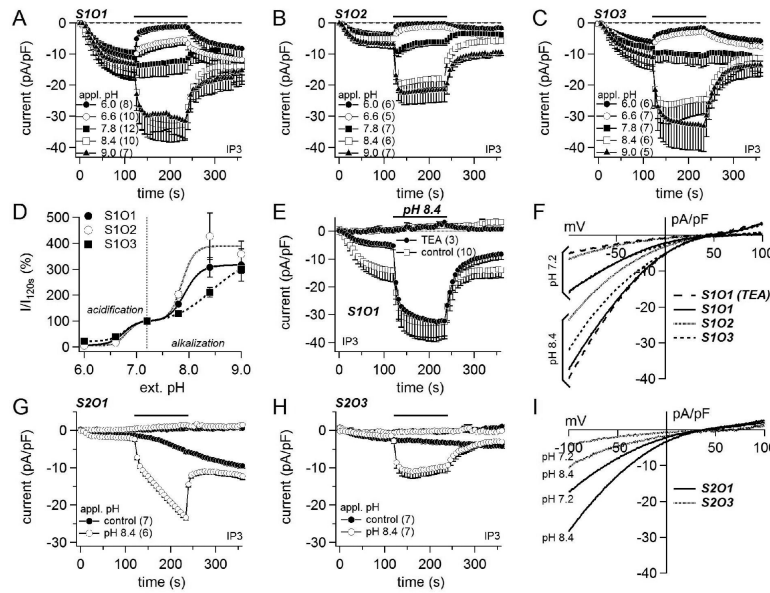
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- $I_{CRAC}$  function depends on intra- and extracellular pH
- Aspartic acids (D110/D112) in Orai1 contribute to the extracellular pH-dependence
- Inhibition of  $Ca^{2+}$  influx by low extracellular pH is a general feature of  $I_{CRAC}$
- Low pH in tumors and infection may thus contribute to dysfunctional  $Ca^{2+}$  signaling



**Fig. 1. Extracellular pH modulates endogenous ICRC**

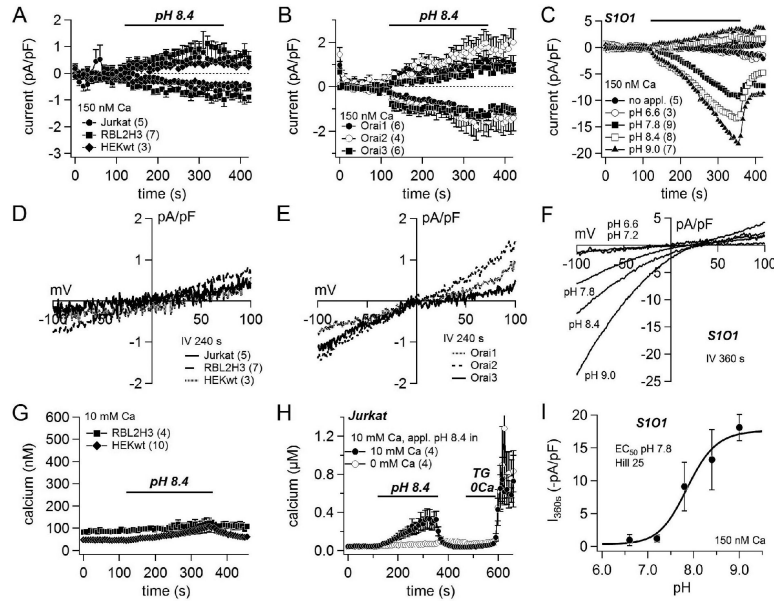
(A, B) Averaged time courses of IP<sub>3</sub>-induced (20 μM) CRAC currents measured in RBL2H3 cells (open circles) and Jurkat T lymphocytes (filled circles). Inward currents of individual cells were measured at -80 mV, normalized by the cell capacitance, and plotted versus time (-SEM). Bars indicate application of external solution of pH 8.4 (A) and 6.0 (B). (C) Inward and outward currents of RBL2H3 cells, measured at -80 mV and +80 mV, respectively, normalized to the amplitude of the inward current right before pH change ( $I/I_{120s}$ ), averaged and plotted versus time ( $\pm$ SEM). The bar indicates application of external solution of different pH (6.0 to 9.0). (D) Double dose response fits (external pH versus relative change of inward current,  $I/I_{120s}$  in %) reveal a  $pK_a$  ( $pK_{acidification}$ ) of 6.8 and  $pK_b$  ( $pK_{alkalization}$ ) of 8.0 in Jurkat T lymphocytes (closed circles) and a  $pK_a$  of 6.7 and  $pK_b$  of 7.8 in RBL2H3 cells (open circles). The number in brackets indicates the number of averaged cells. (E, F) Typical inwardly rectifying I/V relationships of Ca<sup>2+</sup>-selective ICRC at different external pH from RBL2H3 cells (E) and Jurkat T lymphocytes (F).



**Fig. 2. Extracellular pH modulates heterologous STIM/Orai-mediated  $I_{CRAC}$**

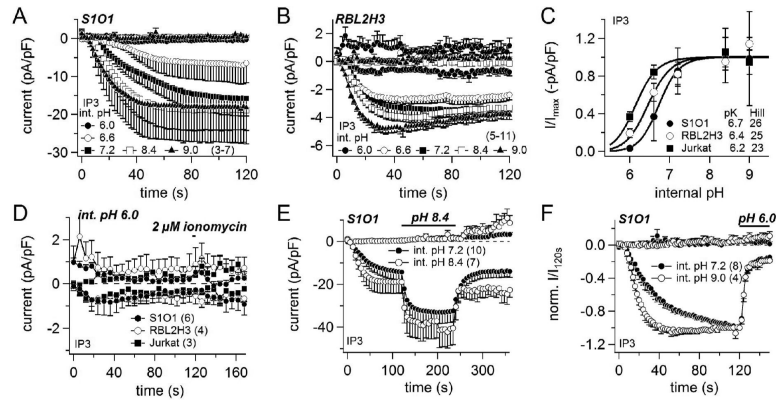
(A, B, C) Averaged time courses of  $IP_3$ -induced ( $20 \mu M$ ) CRAC currents measured in HEK293 cells expressing STIM1 and Orai1 (S1O1; A), STIM1 and Orai2 (S1O2; B) and STIM1 and Orai3 (S1O3; C). Inward currents of individual cells were measured at  $-80$  mV, normalized by the cell capacitance, and plotted versus time ( $\pm$ SEM). The bars indicate application of external solution of different pH (6.0 to 9.0). (D) Double dose response fits (external pH versus relative change of inward current,  $I/I_{120s}$  in %) reveal  $pK_{as}$  ( $pK_{acidification}$ ) of 6.8, 6.8 and 6.7, and  $pK_{bs}$  ( $pK_{alkalization}$ ) of 7.9, 7.9 and 8.4 in S1O1- (filled circles), S1O2- (open circles) and S1O3- (filled boxes) expressing HEK293 cells, respectively. (Same cells as in A-C.) (E) Averaged time courses of  $IP_3$ -induced ( $20 \mu M$ ) inward and outward currents, measured at  $-80$  mV and  $+80$  mV, respectively, in S1O1-expressing HEK293 cells in the presence (open boxes) and absence ( $Na^+$  replaced by  $TEA^+$ ; filled circles) of external sodium. The bar indicates application of external solution of pH 8.4. (F) Typical inwardly rectifying I/V relationships of  $Ca^{2+}$ -selective  $I_{CRAC}$  at different external pH from S1O1-, S1O2- and S1O3-expressing HEK293 cells. The number in brackets indicates the number of averaged cells. (G, H) Averaged time courses of  $IP_3$ -induced ( $20 \mu M$ ) CRAC currents measured in STIM2 and Orai1 (S2O1; G) and STIM2 and Orai3 expressing HEK293 cells (S2O3; H) with (white circles) and without (black circle) application of external solution of pH 8.4. Inward and outward currents of individual cells were measured at  $-80$  mV and  $+80$  mV, respectively, normalized by the cell capacitance, and plotted versus time ( $\pm$ SEM). (I) Typical inwardly rectifying I/V relationships of  $Ca^{2+}$ -selective  $I_{CRAC}$  in different external pH from S2O1- and S2O3-expressing HEK293 cells at different external pH. The number in brackets indicates the number of averaged cells.





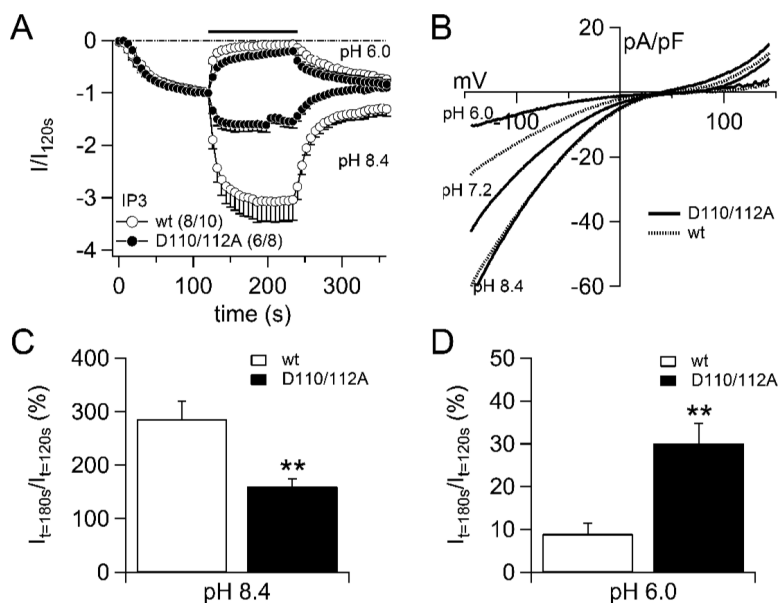
**Fig. 3. Extracellular alkalinization activates heterologous mediated  $I_{CRAC}$**

(A, B) Averaged time courses of external pH 8.4-mediated currents measured in HEK293 wild type (HEK wt; filled diamonds), RBL2H3 cells (filled boxes) and Jurkat T lymphocytes (filled circles) (A). Orai1-(filled circles), Orai2- (open circles) and Orai3-expressing HEK293 cells (filled boxes) (B). Averaged time courses of currents measured in STIM1/Orai1 expressing cells upon application of external solution adjusted to pH values as indicated (C). Inward and outward currents of individual cells were measured at -80 mV and +80 mV, respectively, normalized by the cell capacitance, and plotted versus time ( $\pm$ SEM). The intracellular  $Ca^{2+}$  concentration was clamped to 150 nM (20 mM Cs-BAPTA + 8 mM  $CaCl_2$ ) to prevent passive store depletion-induced CRAC activation. The bars indicate application of external solution of pH as indicated. (D, E, F) I/V relationships corresponding to A, B and C, respectively. (G, H) Averaged time courses of intracellular  $Ca^{2+}$  changes due to application of pH 8.4 in intact Fura-2AM-loaded HEK293 wt cells (open diamonds) and RBL2H3 cells (filled boxes) (G), and Jurkat T lymphocytes in the absence (open circles) and presence (filled circles) of extracellular  $Ca^{2+}$  (H). Bars indicate the application of external solution of pH 8.4 or external solution containing 2  $\mu$ M thapsigargin (TG). (I) Dose-dependence of S1/O1-mediated currents induced by external alkalinization ( $EC_{50}$  pH 7.8). (Same cells as in C.) The number in brackets indicates the number of averaged cells.



**Fig. 4. Intracellular pH modulates IP<sub>3</sub>-induced ICRAC**

(A, B, C) Averaged time courses of IP<sub>3</sub>-induced (20 μM) currents measured in HEK293 cells expressing STIM1 and Orai1 (S1O1; A, C), RBL2H3 cells (B, C) and Jurkat T lymphocytes (C) at different internal pH (6.0 to 9.0). Inward and outward currents of individual cells were measured at -80 mV and +80 mV, respectively, normalized by the cell capacitance, and plotted versus time (±SEM). (C) Dose response fits (internal pH versus current amplitude, normalized to I<sub>max</sub>) reveal pKs of 6.7, 6.4 and 6.2 in S1O1-expressing HEK293 cells (filled circles), RBL2H3 cells (open circles) and Jurkat T lymphocytes (filled boxes), respectively. (Same cells as in A and B.) (D) Averaged time courses of IP<sub>3</sub>-induced (20 μM) currents measured in S1O1-expressing HEK293 cells (filled circles), RBL2H3 cells (open circles) and Jurkat T lymphocytes (filled boxes) at internal pH 6.0. The bar indicates application of 2 μM ionomycin. The number in brackets indicates the number of averaged cells. (E) Averaged time courses of IP<sub>3</sub>-induced (20 μM) CRAC currents at internal pH 7.2 (filled circles) and 8.4 (open circles) measured in STIM1 and Orai1 (S1O1)-expressing HEK293 cells. Inward and outward currents of individual cells were measured at -80 mV and +80 mV, respectively, normalized by the cell capacitance, and plotted versus time (±SEM). The bar indicates application of external solution of pH 8.4. (F) IP<sub>3</sub>-induced inward and outward currents of individual S1O1-expressing HEK293 cells at internal pH 7.2 (filled circles) and 9.0 (open circles), measured at -80 mV and +80 mV, respectively, normalized to the amplitude of the inward current right before pH change (I/I<sub>120s</sub>), averaged and plotted versus time (±SEM). The bar indicates application of external solution of pH 6.0. The number in brackets indicates the number of averaged cells.



**Fig. 5. Contribution of D110 and D112 to pH sensitivity of Orai1**

(A) Averaged time courses of IP<sub>3</sub>-induced (20 μM) CRAC currents measured in HEK293 cells expressing STIM1 and either Orai1 wild type (S1O1; wt) or Orai1<sub>D110/112A</sub>. Inward currents of individual cells were measured at -80 mV. The bars indicate application of external solution of different pH. Currents were normalized to currents at  $t = 120$  s when  $I_{CRAC}$  had fully developed and plotted versus time (-SEM). (B) I/Vs for cells shown in A before application at pH 7.2 and at the end of application ( $t = 180$  s) at pH 6.0 and 8.4. (C) Bar diagram for alkalization induced amplification at  $t = 180$  s. Asterisks indicate a significant difference between Orai1<sub>wt</sub> and Orai1<sub>D110/112A</sub> ( $p = 0.003$  in a two-sided, unpaired student's t-test). (D) Bar diagram for acidification induced residual current. Asterisks indicate a significant difference between Orai1<sub>wt</sub> and Orai1<sub>D110/112A</sub> ( $p = 0.006$  in a two-sided, unpaired student's t-test).