Regulation of Orai1/STIM1 mediated \( I_{CRAC} \) by intracellular pH

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\( \text{Ca}^{2+} \) release activated \( \text{Ca}^{2+} \) (CRAC) channels composed of two cellular proteins, \( \text{Ca}^{2+} \)-sensing stromal interaction molecule 1 (STIM1) and pore-forming Orai1, are the main mediators of the \( \text{Ca}^{2+} \) entry pathway activated in response to depletion of intracellular \( \text{Ca}^{2+} \) stores. Previously it has been shown that the amplitude of CRAC current (\( I_{CRAC} \)) strongly depends on extracellular and intracellular pH. Here we investigate the intracellular pH (pHi) dependence of \( I_{CRAC} \) mediated by Orai1 and STIM1 ectopically expressed in HEK293 cells. The results indicate that pHi affects not only the amplitude of the current, but also \( \text{Ca}^{2+} \) dependent gating of CRAC channels. Intracellular acidification changes the kinetics of \( I_{CRAC} \), introducing prominent re-activation component in the currents recorded in response to voltage steps to strongly negative potentials. \( I_{CRAC} \) with similar kinetics can be observed at normal pHi if the expression levels of Orai1 are increased, relative to the expression levels of STIM1. Mutations in the STIM1 inactivation domain significantly diminish the dependence of \( I_{CRAC} \) kinetics on pHi, but have no effect on pH dependence of \( I_{CRAC} \), amplitude, implying that more than one mechanism is involved in CRAC channel regulation by intracellular pH.

Under normal physiological conditions extracellular pH (pHo) in healthy tissues is maintained within a narrow range between 7.3 and 7.4, while intracellular pH (pHi) is kept between 7.1–7.2\(^1\). In exercising muscle both extracellular and intracellular pH can drop as low as 6.9 and 6.7 respectively\(^2\). In cancerous tumours and wounded tissue, pH variations from the normal values can be even more extreme\(^3\)\(^–\)\(^6\). Dysregulated pH is one of the hallmarks of cancer progression, whereas pH of a wound can be used as a predictor of wound healing outcomes\(^4\)\(^–\)\(^7\). Activity of almost ubiquitously expressed \( \text{Ca}^{2+} \) release activated \( \text{Ca}^{2+} \) (CRAC) channels, formed by Orai1 and STIM1 proteins, has been shown to strongly depend on both extracellular and intracellular pH\(^8\)\(^–\)\(^10\). Considering that CRAC channels have an important role in regulation of immune response, skeletal muscle function and in cancer progression, their dependence on pH is likely to have some physiological significance.

Extracellular acidification inhibits, whereas alkalinisation increases CRAC current (\( I_{CRAC} \)) amplitude with pK\(_a\) of about 8. This has been consistently shown in several publications using both, heterologous expression of Orai1 and STIM1 and cells expressing endogenous \( I_{CRAC} \)\(^8\)\(^–\)\(^11\). The dependence of \( I_{CRAC} \) on extracellular pH in the presence of extracellular \( \text{Ca}^{2+} \) is mediated by E106 in the Orai1 pore, with some contribution from nearby D110 and D112 in the first extracellular loop\(^8\)\(^,\)\(^9\). In the absence of \( \text{Ca}^{2+} \), \( \text{Na}^{+} \) permeability seems to be affected by protonation of E190 residue in the Orai1 pore\(^10\). The mechanism of \( I_{CRAC} \) dependence on intracellular pH is less well understood. Intracellular acidification has been shown to inhibit both, endogenous \( I_{CRAC} \) in different types of cells, and \( I_{CRAC} \) mediated by Orai1 and STIM1 heterologously expressed in HEK293 cells\(^8\)\(^,\)\(^10\)\(^,\)\(^12\). However, intracellular alkalinisation strongly enhanced the amplitude of Orai1/STIM1 mediated \( I_{CRAC} \) in some, but not all studies\(^9\), whereas the amplitude of endogenous \( I_{CRAC} \) in RBL cells and Jurkat T lymphocytes was not affected by pHi rise from 7.4 to 8.4\(^4\)\(^–\)\(^6\).\(^12\).

Theoretically, there are two main mechanisms that may mediate the dependence of \( I_{CRAC} \) function on pH. Protonation/deprotonation of specific residues in Orai1 may affect conductance through the Orai1 pore, and/or protonation/deprotonation of specific residues in Orai1 and/or STIM1 may affect their interaction. The evidence obtained thus far cannot exclude either of these possibilities. Intracellular acidification has been shown to functionally uncouple STIM1 and Orai1 without causing a complete dissociation of STIM1/Orai1 complex, suggesting...
that pH$_i$ affects STIM1/Orai1 interaction$^{13}$. Furthermore, mutation of His155 in the intracellular loop of Orai1 to phenylalanine (H155F) abolishes the effect of alkalinisation on I$_{CRAC}$ and diminishes I$_{CRAC}$ inhibition caused by acidification of pH$_o$$. The Orai1 region containing H155 has previously been implicated in fast Ca$^{2+}$-dependent inactivation (FCDI) of I$_{CRAC}$, and therefore may be involved in STIM1/Orai1 interactions$^{14}$.

In this work, we investigated pH$_i$ dependence of I$_{CRAC}$ mediated by WT Orai1 and WT or mutated STIM1 ectopically expressed in HEK293 cells at two Orai1:STIM1 expression ratios. Using cells expressing WT Orai1 and WT STIM1 we confirmed that I$_{CRAC}$ amplitude strongly depends on pH$_i$ and showed that intracellular acidification introduces a strong re-activation component in I$_{CRAC}$ traces recorded in response to voltage steps between −80 mV and −140 mV. In this study, term “re-activation” is used exclusively in relation to slow increase of I$_{CRAC}$ amplitude during voltage steps from 0 mV to potentials between −80 and −140 mV, and is opposite of FCDI.)

As shown previously, this I$_{CRAC}$ re-activation could also be observed at normal pH$_i$, but only in the cells that were transfected with higher amounts of Orai1 cDNA, relative to STIM1$.^{15,16}$ To investigate whether there is any overlap between the mechanisms that regulate dependence of I$_{CRAC}$ on pH$_i$ and pH$_o$, we used E106D Orai1 mutant. Glutamate 106 in the selectivity centre of Orai pore was previously shown to mediate I$_{CRAC}$ dependence on pH$_i$. In further search for the potential protonation sites that may be responsible for I$_{CRAC}$ dependence on pH$_i$, we evaluated EE482/483AA and DD475/476AA double mutations within STIM1 inactivation domain (ID$_{STIM}$).

Considering that ID$_{STIM}$ is highly negatively charged and is indispensable for Ca$^{2+}$ dependent inactivation of I$_{CRAC}$$^{15,16}$, it is logical to hypothesise that it is involved in pH$_i$ sensitivity of CRAC channel.

### Results

#### Intracellular pH affects CRAC channel gating.

To investigate I$_{CRAC}$ dependence on pH$_i$, the currents were recorded using pipette solutions with pH$_i$ adjusted to 6.3, 7.3 or 8.3. Previously it has been shown that the current amplitude, fast Ca$^{2+}$ dependent inactivation (FCDI), re-activation, potentiation by 2-APB, and selectivity of CRAC channels for divalent cations strongly depend on the relative amounts of Orai1 and STIM1 proteins in the cell$.^{15,16}$ It is possible that other properties of I$_{CRAC}$, including pH$_i$ dependence, are also influenced by the Orai1:STIM1 expression ratios. Therefore, we investigated the effects of pH$_i$ on I$_{CRAC}$ at two transfection conditions. To achieve different expression ratios, HEK293T cells were transfected with Orai1- and STIM1-containing plasmids at either 1:4 or 1:1 molar ratios. For both transfection conditions, the amplitude of I$_{CRAC}$ exhibited strong dependence on pH$_i$. I$_{CRAC}$ was smaller at pH$_i$ 6.3 and larger at pH$_i$ 8.3, compared to pH$_i$ 7.3 (Fig. 1a). Consistent with previous publications, at pH$_i$ 7.3 and 6.3 cells transfected with higher relative amount of STIM1 (1 Orai1: 4 STIM1 ratio) produced larger I$_{CRAC}$ compared to cells transfected with equal amounts of STIM1 and Orai1 (1 Orai1: 1 STIM1 ratio; Fig. 1a).

Majority of cells at pH$_i$ 7.3 produced I$_{CRAC}$ with noticeable FCDI at potentials between −80 and −140 mV when transfected with 1 Orai1: 4 STIM1 ratio (Fig. 1b.i). Some re-activation was also evident with longer pulses (Fig. 1b.ii). It was observed that raising pH$_i$ to 8.3 resulted in elimination of visible signs of the re-activation component, even with longer pulses. However, the extent of FCDI of the currents recorded in response to 200 ms pulses at pH$_i$ 8.3 was also reduced, compared to pH$_i$ 7.3, and the time course of inactivation was significantly slower (c.f. Fig. 1c.i and bi). In contrast, lowering pH$_i$ to 6.3 produced I$_{CRAC}$ with pronounced re-activation at potentials between −80 and −140 mV and no visible FCDI (Fig. 1d).

To compare I$_{CRAC}$ Ca$^{2+}$ dependent gating (FCDI and re-activation) under different conditions and a range of membrane potentials, we used the amplitudes of tail-currents obtained at −100 mV after voltage steps between −140 and +80 mV, normalised to the amplitude of the tail current after a step to +80 mV (see Methods)$^9$. The resulting data were used to construct apparent $P_o$ curves. I$_{CRAC}$ that exhibited FCDI and little or no re-activation produced apparent $P_o$ data that could be fitted with a standard Boltzmann equation (eq. 1), whereas I$_{CRAC}$ with pronounced re-activation exhibited bell-shaped $P_o$ curves which could not be fitted with a single Boltzmann function (Fig. 2a). At the 1Orai1:4STIM1 transfection ratio FCDI was more pronounced at pH$_i$ 7.3 than at pH$_i$ 8.3. At pH$_i$ 6.3, the $P_o$ curve was bell-shaped with a maximum at −20 mV, which was expected, considering the presence of re-activation. However, despite the apparent absence of FCDI in current traces recorded at pH$_i$ 6.3 (Fig. 1d), the FCDI was still present, and the extent of it, relative to the maximum $P_o$ was similar to that of I$_{CRAC}$ recorded at pH$_i$ 7.3 (Fig. 2a).

### Larger I$_{CRAC}$ amplitude at alkaline pH$_i$ is due to pH dependence of EGTA.

Phenomenologically, the effect of intracellular acidification on the I$_{CRAC}$ kinetics and $P_o$ (Figs 1 and 2) was similar to the effect of increasing Orai1 expression relative to STIM1$.^{14}$ I$_{CRAC}$ recorded at pH$_i$ 7.3 in the cells transfected with 1Orai1:1STIM1 ratio showed strong re-activation during voltage steps from 0 mV to −120 mV and produced bell-shaped $P_o$ curve, which looked similar to the $P_o$ curve obtained at pH$_i$ 6.3 with 1 Orai1: 4 STIM1 transfection ratio (c.f. Fig. 2a and b). Lowering pH$_i$ to 6.3 in cells transfected with 1 Orai1: 1 STIM1 ratio further increased the re-activation (Fig. 2c). In contrast, raising pH$_i$ to 8.3 virtually eliminated current re-activation (Fig. 2c). The apparent $P_o$ curves obtained at pH$_i$ 6.3 in cells transfected with 1:1 and 1:4 Orai1:STIM1 ratios were almost identical between two transfection conditions (Fig. 2d). The observed changes in the kinetics and the extent of I$_{CRAC}$ FCDI induced by raising pH$_i$ to 8.3 (Fig. 1c) are similar to those caused by replacing EGTA with BAPTA at pH$_i$ 7.3.$^3,18,20$ Due to its’ ability to bind Ca$^{2+}$ faster than EGTA, BAPTA is believed to reduce Ca$^{2+}$ concentration at the intracellular mouth of CRAC channels, thus slowing down and reducing FCDI$^{18,20}$. The apparent $P_o$ curve obtained at pH$_i$ 7.3 using cells transfected with 1 Orai1: 4 STIM1 ratio and BAPTA as Ca$^{2+}$ buffer, was virtually identical to $P_o$ curves obtained at pH$_i$ 8.3 and EGTA in the pipette solution (Fig. 2d). Using BAPTA in the internal solution instead of EGTA with pH$_i$ 6.3 also decreased I$_{CRAC}$ re-activation at negative potentials and therefore reduced positive apparent $P_o$ (Fig. 2d).
Figure 1. The dependence of $I_{\text{CRAC}}$ amplitude and kinetics on pH$_i$. (a) Current density at $-100$ mV, obtained from instantaneous $I$–$V$ plots in response to 100 ms ramps between $-120$ and 120 mV recorded after a complete development of $I_{\text{CRAC}}$ at indicated pH$_i$ and Orai1:STIM1 transfection ratios. The amplitude of $I_{\text{CRAC}}$ at pH$_i$ 7.3 was significantly different from the amplitudes at pH$_i$ 6.3 and 7.3 at both transfection ratios (P < 0.001; unpaired $t$-test). The amplitudes of $I_{\text{CRAC}}$ at 1Orai1:1STIM1 transfection ratio was significantly smaller that the amplitudes at 1Orai1:4STIM1 ratio at pH$_i$ 6.3 and 7.3 (P < 0.03; unpaired $t$-test), but not at pH$_i$ 8.3. (b,c and d) The examples of WT $I_{\text{CRAC}}$ traces at pH$_i$ 7.3, 8.3 and 6.3, correspondingly. Currents were recorded in HEK293T cells transfected with Orai1 and STIM1 at 1:4 ratio, in response to 200 ms (i) and 600 ms (ii) voltage steps from 0 mV holding potential to $-120$ mV.
To investigate whether intracellular Ca\(^{2+}\) buffer contributes to the dependence of I\(_{\text{CRAC}}\) on pH\(_i\), we used extracellular application of 30 mM NH\(_4\)Cl, which is known to alkalinise pH\(_i\)13, 21. Application of NH\(_4\)Cl to the bath, when EGTA was used as Ca\(^{2+}\) buffer in the pipette solution, drastically increased the I\(_{\text{CRAC}}\) amplitude (Fig. 3a, b) and caused inhibition of both I\(_{\text{CRAC}}\) FCDI and re-activation (Fig. 3c), in agreement with the results obtained using pipette solution with EGTA and pH\(_i\) 8.3 (Figs 1a and 2a, c). In contrast, application of NH\(_4\)Cl when BAPTA was used in the pipette solution instead of EGTA, had very little effect on I\(_{\text{CRAC}}\) amplitude (Fig. 3a, b).

Is there any overlap between mechanisms regulating I\(_{\text{CRAC}}\) dependence on pH\(_o\) and pH\(_i\)?

Previous investigations have shown that the amplitude of native I\(_{\text{CRAC}}\) in different cell types and I\(_{\text{CRAC}}\) mediated by heterologously expressed Orai1 and STIM1 strongly depends on extracellular pH\(_o\)8–10, 12. Superficially, the dependence of I\(_{\text{CRAC}}\) amplitude on pH\(_o\) looks similar to its dependence on pH\(_i\)8–10, 12. However, possible reasons for similarities between pH\(_i\) and pH\(_o\) effects on I\(_{\text{CRAC}}\) have not been yet considered. Could changing pH\(_o\) affect pH\(_i\) in patch clamping experiments? To investigate this question, we used cells transfected with Orai1 and STIM1 at 1:1 molar ratio, which showed a very pronounced re-activation at negative potentials (Fig. 4a). Raising pH\(_i\) to 8.3 eliminates I\(_{\text{CRAC}}\) re-activation at negative potentials (Fig. 2b), and if raising pH\(_i\) results in a rise of pH\(_o\), one would expect a reduction of current re-activation. The results show that increasing pH\(_o\) from 7.4 to 8.3 does not reduce I\(_{\text{CRAC}}\) re-activation and has no effect on the P\(_o\) curve (Fig. 4). Therefore, it can be safely concluded that pH\(_i\) in these patch clamping experiments is not affected by changes in pH\(_o\).
One of the main residues responsible for ICRAC dependence on pHo is Glu106 in the Orai1 pore. Could pHi affect protonation of Glu106 in the Orai1 pore? Despite the observation that ICRAC kinetics is unaffected by pHo, it is possible that ICRAC amplitude dependence on pHi and pHo is mediated by the same protonatable site in the pore. To investigate this possibility, we used an E106D Orai1 mutant. Previous studies have shown that the E106D Orai1 differs from WT Orai1 in several respects. Firstly, it is less selective for Ca2+ and supports a significant Na+ conductance. Secondly, while E106D-mediated ICRAC exhibits strong inactivation at negative potentials that looks similar to FCDI of WT ICRAC (Fig. 5a, cf. Fig. 1b), it has a different underlying mechanism. The inactivation of E106D-mediated ICRAC during steps to negative potentials is caused by Ca2+ block of Na+ permeation through the pore; it does not require interaction with IDSTIM, and it is not affected by BAPTA or Orai1:STIM1 transfection ratios. Finally, and importantly for this investigation, the Ca2+ dependent block of Na+ permeation through E106D Orai1 is strongly pHo dependent, whereas the peak amplitude of ICRAC mediated by E106D Orai1 is not influenced by pHo. Changing the pipette solution pH revealed that the amplitude of E106D-mediated ICRAC was pHi-dependent – the current was strongly inhibited by pHi, 6.3 and enhanced by pHi, 8.3, similarly to WT ICRAC (Fig. 5c, cf. Fig. 1a). E106D-mediated ICRAC recorded in the absence of Na+ in the bath solution, when Ca2+ was the only permeating cation, also showed pHi dependence of the amplitude similar to that of WT ICRAC (Fig. 5d). However, the kinetics and the extent of Ca2+ dependent block of Na+ permeation through E106D Orai1 was not appreciably affected by pHi (Fig. 5b, cf. Figs 5a and 1d). If Asp 106 could be protonated from the intracellular side at low pHio, one would expect the changes in E106D-mediated ICRAC to be similar to those induced by low pHi.
which was not the case. These results demonstrate that pH$_i$ and pH$_o$ affect I$_{CRAC}$ through different mechanisms, and that Glu 106 which is located in the Orai1 pore does not mediate the pH$_i$-dependence of I$_{CRAC}$ amplitude.

The effects of mutations in STIM inactivation domain on I$_{CRAC}$ dependence on pH$_i$. One of the domains within STIM1/Orai1 complex critically important for FCDI is located on STIM1 between residues 470 and 491 (ID$_{STIM}$), C-terminal to CRAC activation domain (CAD)\cite{17,18}. Thus, neutralisation of Aspartate and Glutamate residues within a cluster of 7 negatively charged amino acids (475DDVDDMDEE483) in ID$_{STIM}$ results in drastic changes in I$_{CRAC}$ FCDI\cite{18}. It is possible that protonation/deprotonation of some of these residues contribute to I$_{CRAC}$ dependence on pH$_i$. To investigate this possibility, we investigated pH$_i$ dependence of two double mutants of STIM1, DD475/476AA, which produced I$_{CRAC}$ with diminished FCDI (Fig. 6a,c), and EE482/483AA, which produced I$_{CRAC}$ with enhanced FCDI (Fig. 6b,c)\cite{17,18}. Using pipette solutions with pH adjusted to 6.3, 7.3 or 8.3 we found that the amplitude of I$_{CRAC}$ mediated by each of these STIM1 mutants co-expressed with WT Orai1 exhibited dependence on pH$_i$ similar to that of WT I$_{CRAC}$ (Fig. 6d; cf. Fig. 1a).

Next, we investigated the effects of DD475/476AA and EE482/483AA STIM1 mutations on the dependence of I$_{CRAC}$ kinetics on pH$_i$. At the transfection ratio of 1 Orai1: 1 STIM1 the apparent $P_o$ for I$_{CRAC}$ mediated by DD475/476AA-STIM1 mutant showed a weaker dependence on pH$_i$ compared to WT I$_{CRAC}$ (Fig. 7a; cf. Fig. 2a). Although pH 8.3 reduced the re-activation component (Fig. 7a), as it did in WT I$_{CRAC}$ (Fig. 2a), pH 6.2 failed to induce a significant change in the apparent $P_o$ of the Orai1/ DD475/476AA-STIM1 mediated current (Fig. 7a). Changing the transfection ratio to 1 Orai1: 1 DD475/476AA-STIM1 did not affect the apparent $P_o$, or its dependence on pH$_i$ (Fig. 7b). However, we were unable to obtain the apparent $P_o$ curve at pH 6.3 as the amplitude of the current was too small for a reliable extraction of the data.

Figure 4. Extracellular alkalinisation has no effect on I$_{CRAC}$ kinetics. (a,b) Examples of I$_{CRAC}$ traces recorded in response to −120 mV steps in the bath solution of pH$_o$ 7.4 and after changing pH$_o$ to 8.3, correspondingly. (c) Apparent $P_o$ curves at pH$_i$ 7.4 (filled circles) and after changing pH$_o$ to 8.3 (clear circles) (n = 4). HEK293T cells were transfected with Orai1 and STIM1 plasmids at 1:1 molar ratio. pH of the pipette solution was 7.3.
ICRAC mediated by Orai1 and EE482/483AA-STIM1 mutant also exhibited a weaker dependence of the kinetics on pH_i, compared to WT CRAC (Fig. 7c,d). At the transfection ratio 1 Orai1: 4 EE482/483AA-STIM1, lowering pH_i to 6.3 introduced a small re-activation component to the current (Fig. 7c). This can be seen on P_o curve as deviation from simple Boltzmann distribution, whereas increasing pH_i to 8.3 slightly reduced the extent of FCDI at negative potentials (Fig. 7c). At the transfection ratio 1:1, the changes in FCDI and re-activation induced by changes in pH_i were more pronounced than at the ratio 1:4 (Fig. 7d, cf. Fig. 7c), however, these changes were significantly smaller than those induced by pH_i changes in the WT I_CrAC (Fig. 2a,c; cf. Fig. 7c,d).

Overall, DD475/476AA and EE482/483AA STIM1 double mutations significantly diminished the dependence of I_CrAC FCDI on pH_i and the relative Orai1/STIM1 expression ratio, without affecting pH_i dependence of I_CrAC amplitude.

One of the distinctive properties of Orai1/STIM1 mediated I_CrAC is inhibition by high (over 100 µM) and potentiation by low (below 10 µM) concentrations of 2-APB, whereas application of intermediate concentrations of 2-APB (10–50 µM) cause transient potentiation of I_CrAC followed by inhibition 22. Previously we have shown that the extent of I_CrAC potentiation by 2-APB depends on the relative expression levels of STIM1 and Orai1 16. Here we investigated whether potentiation of I_CrAC amplitude by 50 µM 2-APB is affected by pH_i. The amplitude of I_CrAC in cells transfected with WT STIM1 and Orai1 at 4:1 ratio increased more than 4-fold at acidic pH_i of 6.3, but only 1.3-fold when pH_i was raised to 8.3, compared to a potentiation of 2.5-fold at pH 7.3 (Fig. 8a). Despite the lack of pH_i effect on FCDI and the apparent P_o of I_CrAC mediated by Orai1/EE482/483AA-STIM1, the dependence of 2-APB mediated potentiation of this mutant on pH_i remained unchanged, compared to WT I_CrAC (Fig. 8b).

**Discussion**

The key findings of this paper can be summarised as follows – (i) pH_i regulates both, the amplitude of I_CrAC and Ca^{2+} dependent gating of CRAC channels; (ii) increase in I_CrAC amplitude in response to alkaline pH_i in the
presence of EGTA in the pipette solution is a result of pH dependence of the Ca\textsuperscript{2+} buffering properties of EGTA, not the CRAC channel itself; (iii) Glutamate 106 in the selectivity centre of Orai1 pore, which mediates I\textsubscript{CRAC} dependence on pH\textsubscript{i}; does not contribute to I\textsubscript{CRAC} dependence on pH\textsubscript{i}; (iv) negatively charged residues in ID\textsubscript{STIM} domain play a role in pH\textsubscript{i} regulation of CRAC channel gating kinetics but not the amplitude of I\textsubscript{CRAC}. These data suggest that several mechanisms contribute to I\textsubscript{CRAC} regulation by pH\textsubscript{i}.

It has been shown previously that increasing the amounts of Orai1 relative to STIM1 results in a smaller I\textsubscript{CRAC} that exhibits re-activation at negative potentials which masks FCDI\textsuperscript{15, 16}. The results presented here show that intracellular acidification has an effect on I\textsubscript{CRAC} similar to that of increasing the relative amounts of Orai1 (or decreasing the relative amounts of STIM1). Comparable changes in I\textsubscript{CRAC} kinetics and amplitude caused by intracellular acidification and increased Orai1:STIM1 ratio suggest that low pH\textsubscript{i} reduces the affinity of STIM1 binding to Orai1, likely due to protonation of specific residues, which is equivalent to a reduction of available STIM1. This notion is supported by previous observations that acidification of cytoplasm due to hypoxia reduces FRET between Orai1 and STIM1 and inhibits I\textsubscript{CRAC}\textsuperscript{13}. In the study of Mancarella et al. (2012) the effect of hypoxia on I\textsubscript{CRAC} could be mimicked by application of extracellular propionate, which lowers pH\textsubscript{i}, and reversed by application of NH\textsubscript{4}Cl, which raises pH\textsubscript{i}\textsubscript{13}. Intracellular acidification was shown to reduce FRET between STIM1-YFP and Orai1-CFP, but no change was observed in STIM1/Orai1 co-localisation in puncta\textsuperscript{13}. These results suggested that pH\textsubscript{i} affects STIM1/Orai1 functional coupling leading to channel opening, but not the interactions that trap STIM1 and Orai1 in puncta\textsuperscript{13}. The pH dependent changes in I\textsubscript{CRAC} kinetics reported here also point to the conclusion that intracellular acidification disrupts STIM1/Orai1 functional interactions.

**Figure 6.** The dependence of I\textsubscript{CRAC} amplitude, mediated by Orai1 and DD475/6AA and EE482/3AA STIM1 mutants, on pH\textsubscript{i}. (a,b) Examples of I\textsubscript{CRAC} traces recorded in response to 200 ms voltage steps from 0 mV to −120 mV in cells transfected with Orai1 and either DD475/6AA STIM1 (a) or EE482/3AA STIM1 (b) at pH\textsubscript{i} 7.3. (c) Apparent P\textsubscript{o} curves obtained at pH\textsubscript{i} 7.3 using cells transfected with Orai1 and DD475/6AA STIM1 (filled circles), Orai1 and WT STIM1 (clear circles), and Orai1 and EE482/3AA STIM1 (clear triangles), at 1:4 ratio. (d) ID\textsubscript{STIM} mutants I\textsubscript{CRAC} amplitude was measured at −100 mV from the responses to 100 ms voltage ramps from −120 to 120 mV, at indicated pH\textsubscript{i}. HEK293T cells were transfected with Orai1 and DD475/6AA STIM1 or EE482/3AA STIM1 plasmids at 1:4 ratio.
Inhibition of ICRAC by low pH has been demonstrated previously in several publications. They all agree that ICRAC, both endogenous and mediated by ectopically expressed Orai1 and STIM1, is inhibited by approximately 70–90% at pH of around 6, compared to pH 7.3. In contrast, the effects of alkalinisation of pH above 7.3 on ICRAC are inconsistent between different studies. The results of the present work suggest that the reason for the discrepancy is likely to be due to the type of intracellular Ca\(^{2+}\) buffer used. Studies employing BAPTA in the pipette did not find much increase in ICRAC amplitude at higher pH, whereas studies that used EGTA reported a significant potentiation of ICRAC amplitude by alkalinisation. Calculations using Maxchelator indicate that Ca\(^{2+}\) buffering capacity of EGTA is highly pH dependent, and raising pH by one unit increases EGTA binding affinity to Ca\(^{2+}\) two orders in magnitude, changing K\(_d\) from 1.28 \times 10^{-7} M at pH 7.3 to 1.4 \times 10^{-9} M at pH 8.3, whereas pH dependence of Ca\(^{2+}\) buffering by BAPTA is weak.

The observations reported here which show strong increase in ICRAC amplitude in response to NH\(_4\)Cl application to the bath when EGTA is used in the pipette, and virtual absence of such effect when intracellular Ca\(^{2+}\) is buffered with BAPTA, suggest that the Ca\(^{2+}\) binding properties of EGTA play a significant part in ICRAC pH dependence in the presence of EGTA, particularly, when pH rises above 7.5. The increase in ICRAC amplitude at alkaline pH is likely to be due to stronger and faster Ca\(^{2+}\) binding by EGTA, rather than increase in pH per se. pH dependence of EGTA Ca\(^{2+}\) binding properties creates unwanted complications for the interpretation of the experimental results. However, many physiological intracellular Ca\(^{2+}\) buffers are likely to exhibit strong pH dependence, similarly to EGTA. This is supported by the observations that intracellular alkalinisation induced by application of NH\(_4\)Cl to the bath in Ca\(^{2+}\) imaging experiments, when cells have endogenous intracellular Ca\(^{2+}\) buffering, potentiates store-operated Ca\(^{2+}\) entry in platelets and HT-29 cells. Therefore, results obtained using EGTA, rather than BAPTA, may have more physiological relevance. Much bigger amplitude of ICRAC activated by IP\(_3\) in the presence of BAPTA in the pipette solution, compared to EGTA, was noticed very early on. However, the reason for this difference remains poorly understood.

The only residue that has been implicated in ICRAC dependence on pH so far is His 155 in Orai1. H155F mutation in Orai1 was shown to abolish the increase of ICRAC amplitude in response to intracellular alkalinisation, but ICRAC mediated by H155F-Orai1 was still inhibited by about 60% at low pH, which implies that His 155 is
unlikely to be the only site that mediates I_{CRAC} regulation by pH_i. Data presented in this work indicate that Glut106 in the Orai1 selectivity centre, which can be protonated from the extracellular side⁹, does not contribute to pH_i dependence at all, which also suggests that Orai1 pore is not permeable to protons. Presence of seven negatively charged residues within ID_{STIM} and the fact that neutralisation of three of them, D476, D478, and D479, significantly reduced the FCDI, similarly to acidic pH_i, made ID_{STIM} a good candidate for the pHi sensor of CRAC channel¹⁸. The results presented here indicate that ID_{STIM} is not involved in pH_i dependence of the I_{CRAC} amplitude, but mutations in ID_{STIM} affect pH_i regulation of I_{CRAC} Ca^{2+} dependent gating. The kinetics of I_{CRAC} mediated by Orai1/EE482/483AA-STIM1 or Orai1/DD475/476AA-STIM1 was not appreciably affected by either acidic, or alkaline pH_i. It is unlikely, however, that protonation/deprotonation of negatively charged resides in ID_{STIM} is responsible for the changes in I_{CRAC} kinetics induced by the changes in pH_i. Neutralisation of aspartates 482 and 483 increases FCDI, so protonation of these aspartates alone cannot be responsible for reduced FCDI at acidic pH_i. It has been shown previously that neutralization of these aspartates together with glutamates in ID_{STIM} reduce FCDI, i.e., the effect of neutralisation of glutamates overcomes the effect of neutralisation of aspartates. This suggests that if glutamates in the ID_{STIM} were protonated at acidic pH_i, Orai1/EE482/483AA-STIM1 would display dependence of kinetics on pH_i, similar to that of WT I_{CRAC}. However, this was not the case, which excludes ID_{STIM} as a direct pH_i sensor.

Interestingly, EE482/3AA-STIM1 significantly diminished the dependence of I_{CRAC} kinetics not only on pH_i, but also on the relative expression levels of STIM1 and Orai1. This could've been a result of saturating levels of STIM1 in buffer at Orai1 due to changes in pH_i or moderate increase in Orai1 expression, are unlikely to have an appreciable effect on I_{CRAC} kinetics. However, when the amounts of STIM1 are close to saturating, 2-APB does not potentiate I_{CRAC}. Application of 2-APB to Orai1 EE482/483AA-STIM1 mediated I_{CRAC} caused the same level of potentiation as in WT I_{CRAC} at all intracellular pH tested. This indicates that expression levels of mutant STIM1 were not different from that of WT STIM1, and that pH_i affected functional coupling of Orai1 with mutant STIM1 in the same way it has affected it’s functional coupling with WT STIM1. The lack of the dependence of Orai1 EE482/483AA-STIM1 I_{CRAC} kinetics on the Orai1:STIM1 relative expression ratio and pH_i suggests that the minimum number of this mutant STIM1 peptides which is needed to open Orai1 pore, is sufficient to support fully functional I_{CRAC} FCDI.

In conclusion, the results presented here support the hypothesis that I_{CRAC} inhibition by intracellular acidification is caused by disruption of functional coupling of STIM1 and Orai1, whereas the increase in I_{CRAC} amplitude at alkaline pH_i in the presence of EGTA is mainly due to increased Ca^{2+} buffering capacity of EGTA. Negatively charged ID_{STIM} is not a direct pH_i sensor, but mutations neutralising negative charges in ID_{STIM} affect pH_i dependence of I_{CRAC} kinetics by changing the interaction between STIM1 and Orai1.

**Methods**

**Cell culture and transfections.** HEK-293T cells [human embryonic kidney-293 cells expressing the large T antigen of SV40 (simian virus 40)] (A.T.C.C. CRL 11268) were cultured at 37 °C in 5% (v/v) CO₂ in air in DMEM (Dulbecco’s modified Eagle’s medium) supplemented with 100 μM nonessential amino acids, 2 mM L-glutamine and 10% fetal bovine serum⁹,¹⁶. To co-express WT Orai1 with WT STIM1 or double STIM1 mutants (EE482/483AA and DD475/476AA), cells seeded on glass cover slips were transfected using Polyfect (Qiagen).
transfection reagent according to the manufacturer’s instructions. The Orai1 and STIM1 (WT or mutant) plasmids were transfected at two Orai1:STIM1 molar ratios 1:1 and 1:4.15-16. Plasmids containing EE482/483AA and DD475/476AA double STIM1 mutants were generously provided by Prof Richard Lewis (Stanford University, USA).

**Patch clamping.** Whole-cell patch clamping was performed at room temperature (23°C) using a computer based patch-clamp amplifier (EPC-9, HEKA Elektronik) and PULSE software (HEKA Elektronik) as previously described.17-19 The control bath solution contained 140 mM NaCl, 4 mM CaCl2, 10 mM MgCl2, and 10 mM HEPES adjusted to pH 7.4 with NaOH. Depletion of intracellular Ca2+ stores was achieved using 20 μM Ins(3,4,5)P3 (Sigma) added to an internal solution containing 130 mM caesium glutamate, 10 mM CaCl2, 5 mM MgCl2, 1 mM MgATP, 10 mM EGTA and either 10 mM MES adjusted to pH 6.3 with NaOH, or 10 mM HEPES adjusted to pH 7.3 or 8.3 with NaOH. Patch pipettes were pulled from borosilicate glass and fire polished to give a pipette resistance between 2 and 4 MΩ. Series resistance did not exceed 15 MΩ and was 50-70% compensated. Traces obtained before activation of ICRAC, or after its inhibition with 10 μM La3+ were used for leakage subtraction.

**Data analysis.** To obtain apparent (relative) open probability (PR) curves of CRAC channels, instantaneous tail currents recorded in response to voltage steps to −100 mV after test pulses between −140 and 80 mV, applied every 5 in 20 mV increments, were normalised to the amplitude of the instantaneous tail current recorded after test pulse to 80 mV and plotted against corresponding test pulse voltage.9 The length of the test pulses was set to 150 ms to make sure that both gating processes of ICRAC—inactivation and re-activation are captured in one protocol. Were possible, the data points were fitted with the Boltzmann distribution with an offset of the form:

\[
P_o(V) = P_{\text{min}} + \frac{1 - P_{\text{min}}}{1 + \exp((V_{1/2} - V)/k)}
\]

where \(P_{\text{min}}\) is an offset, \(V\) is the membrane potential, \(V_{1/2}\) is the half-maximal activation potential (\(V_{1/2}\) corresponds to the inflexion point of the \(P_o\) curve) and \(k\) is the slope factor. However, in many cases apparent \(P_o\) data could not be fitted with Boltzmann distribution and the data points were fitted with a smooth curve using cubic spline procedure in Prizm 6 software.

**References**

1. Boron, W. F. & Boulpaep, E. L. Medical Physiology: A Cellular and Molecular Approach. (Elsevier/Saunders, 2009).
2. Street, D., Bangsbo, J. & Juel, C. Interstitial pH in human skeletal muscle during and after dynamic graded exercise. *Wound Repair and Regeneration* 22, 174–186 (2014).
3. De Milito, A. & Fais, S. Tumor acidity, chemoresistance and proton pump inhibitors. *Future Oncology* 1, 779–786 (2005).
4. Percival, S. L., McCarty, S., Hunt, J. A. & Woods, E. J. The effects of pH on wound healing, biofilms, and antimicrobial efficacy. *Wound Repair and Regeneration* 22, 174–186 (2014).
5. Scrimgeour, N. R., Wilson, D. P. & Rychkov, G. Y. Glutamate 106 in the Orai1 pore contributes to fast Ca2+-dependent inactivation of Ca2+ release-activated Ca2+ (CRAC) current. Biochem J 411, 743–753 (2012).
6. Taylor, S. R. Microenvironment acidity as a major determinant of tumor chemoresistance: Proton pump inhibitors (PPIs) as a novel therapeutic approach. *Drug Resistance Updates* 23, 69–78 (2015).
7. Glitsch, M. Protons and Ca2+: Ionic Allies in Tumor Progression? *Physiology* 26, 252–265 (2011).
8. Beck, A., Fleig, A., Penner, R. & Peinelt, C. Regulation of endogenous and heterologous Ca2+ release-activated Ca2+ currents by pH. *Cell Calcium* 56, 235–243 (2014).
9. Scrimgeour, N. R., Wilson, D. P. & Rychkov, G. Y. Glutamate 106 in the Orai1 pore contributes to fast Ca2+-dependent inactivation and re-activation of CRAC channels. *J Gen Physiol* 147, 153–164 (2016).
10. Mullins, F. M. & Lewis, R. S. The inactivation domain of STIM1 is functionally coupled with the Orai pore to enable Ca2+-dependent inactivation. *J Gen Physiol* 147, 153–164 (2016).
11. Mullins, F. M., Park, C. Y., Dolmetsch, R. E. & Lewis, R. S. STIM1 and calmodulin interact with Orai1 to induce Ca2+-dependent inactivation of CRAC channels. *Proc Natl Acad Sci USA* 106, 15495–15500 (2009).
12. Thromb Haemost 90, 1121–1127 (2003).
13. Prakriya, M. & Lewis, R. S. Potentiation and inhibition of Ca2+ release-activated Ca2+ channels by 2-aminoethyldiphenyl borate (2-APB) occurs independently of IP3 receptors. *J Physiol* 536, 3–19 (2001).
14. Schwalger, B. Cytosolic Ca2+ buffers. *Cold Spring Harb Perspect Biol* 2, a004051 (2010).
15. Kesvatera, T., Jonsson, B., Thulin, E. & Linse, S. Focusing of the electrostatic potential at EF-hands of calbindin D-9k: Titration of acidic residues. *Proteins* 45, 129–135 (2001).
16. Nitschke, R. et al. The effect of intracellular pH on cytosolic Ca2+ in HT29 cells. *Pflugers Archiv: European journal of physiology* 433, 98–108 (1996).
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Author Contributions
D.G., N.R.S., S.G., and L.M. carried out patch clamping experiments and contributed to the analysis of the data. F.H.Z. and G.J.B. contributed to experimental design and interpretation of the data. G.Y.R. conceived and supervised the work. G.Y.R. and G.J.B. wrote the paper. All authors contributed to final approval of the manuscript prior to submission.

Additional Information
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