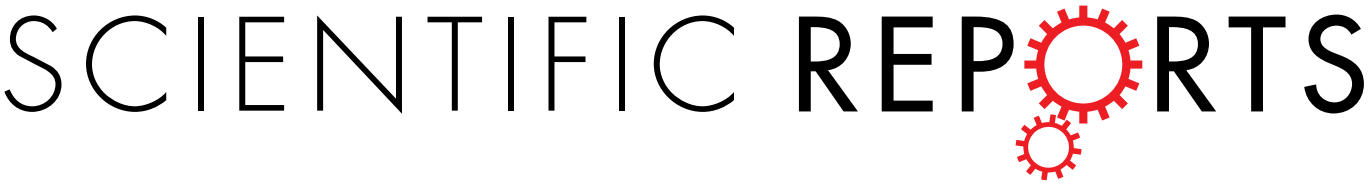

OPEN

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

D. Gavriliouk[1], N. R. Scrimgeour[1], S. Grigoryev[2], L. Ma[1,4], F. H. Zhou[2], G. J. Barritt[3] & G. Y. Rychkov[1,2]

Received: 24 January 2017
Accepted: 12 June 2017
Published online: 29 August 2017

Ca^{2+} release activated Ca^{2+} (CRAC) channels composed of two cellular proteins, Ca^{2+}-sensing stromal interaction molecule 1 (STIM1) and pore-forming Orai1, are the main mediators of the Ca^{2+} entry pathway activated in response to depletion of intracellular 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 (pH_i) dependence of I_{CRAC} mediated by Orai1 and STIM1ectopically expressed in HEK293 cells. The results indicate that pH_i affects not only the amplitude of the current, but also 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 pH_i 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 pH_i, but have no effect on pH_i 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 (pH_o) in healthy tissues is maintained within a narrow range between 7.3 and 7.4, while intracellular pH (pH_i) 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 Ca^{2+} release activated 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 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 Ca^{2+}, 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[10], whereas the amplitude of endogenous I_{CRAC} in RBL cells and Jurkat T lymphocytes was not affected by pH_i rise from 7.4 to 8.4[8,12].

Theoretically, there are two main mechanisms that may mediate the dependence of I_{CRAC} function on pH_i. 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

[1]School of Medical Sciences, University of Adelaide, Adelaide, South Australia, 5005, Australia. [2]School of Medicine, University of Adelaide, and South Australian Health and Medical Research Institute, Adelaide, South Australia, 5005, Australia. [3]School of Medicine, Flinders University of South Australia, Bedford Park, South Australia, 5065, Australia. [4]Present address: Institute for Molecular Bioscience, University of Queensland, Brisbane, QLD 4072, Australia. Dan Gavriliouk and Nathan R Scrimgeour contributed equally to this work. Correspondence and requests for materials should be addressed to G.Y.R. (email: Grigori.Rychkov@adelaide.edu.au)

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_i[10]. 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[9,15,16]. 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 Orai1 pore was previously shown to mediate I_{CRAC} dependence on pH_o[9]. 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}[17,18], 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 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). However, the effect of the relative expression ratio on the current amplitude, at least within the employed transfection range (1:4 and 1:1), was absent when pH_i was raised to 8.3 (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. 1ci 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[9]. 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[16]. 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, rising pH_i to 8.3 virtually eliminated current re-activation (Fig. 2c). The apparent P_o curves obtained at pH_i 8.3 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[9,19,20]. 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[19,20]. 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).

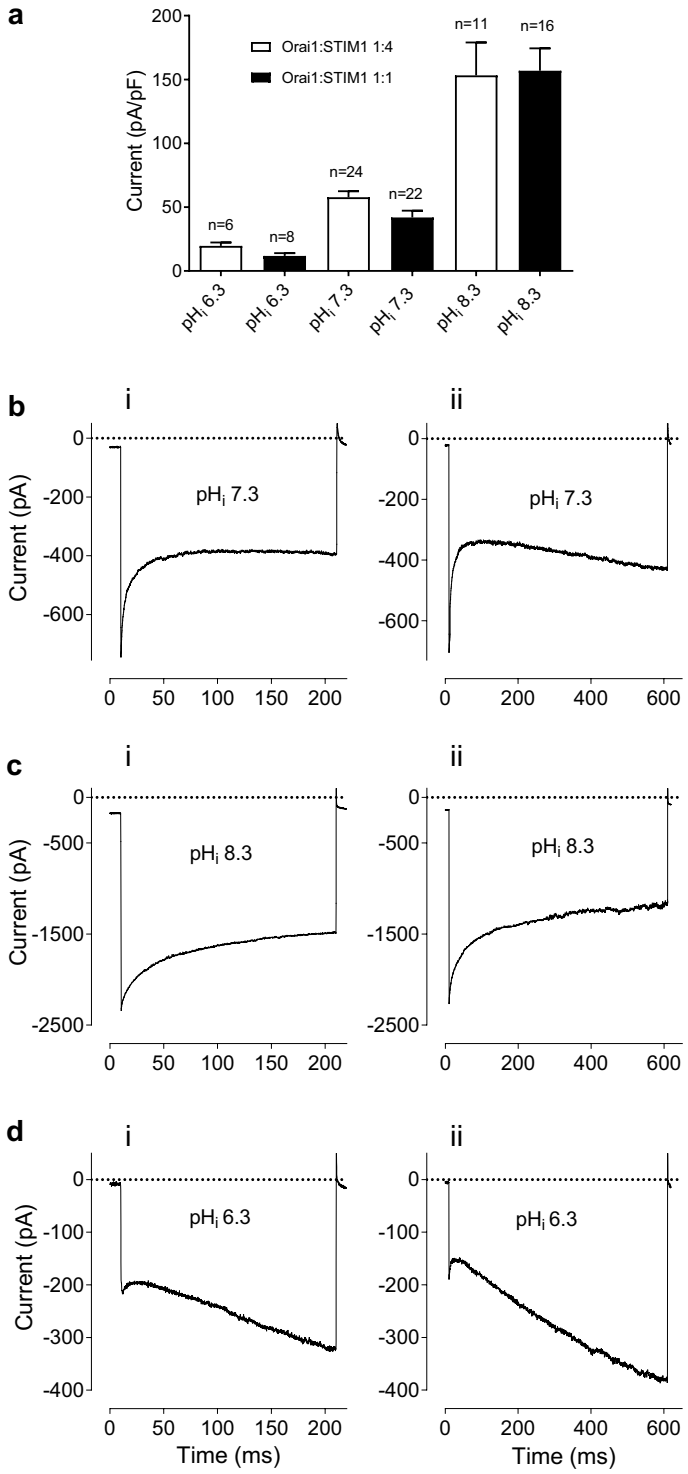

Figure 1. The dependence of I_{CRAC} amplitude and kinetics on pH_i. (**a**) Current density at −100 mV, obtained from instantaneous I–V plots in response to 100 ms ramps between −120 and 120 mV recorded after a complete development of I_{CRAC}, at indicated pH_i and Orai1:STIM1 transfection ratios. The amplitude of I_{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_{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_{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.

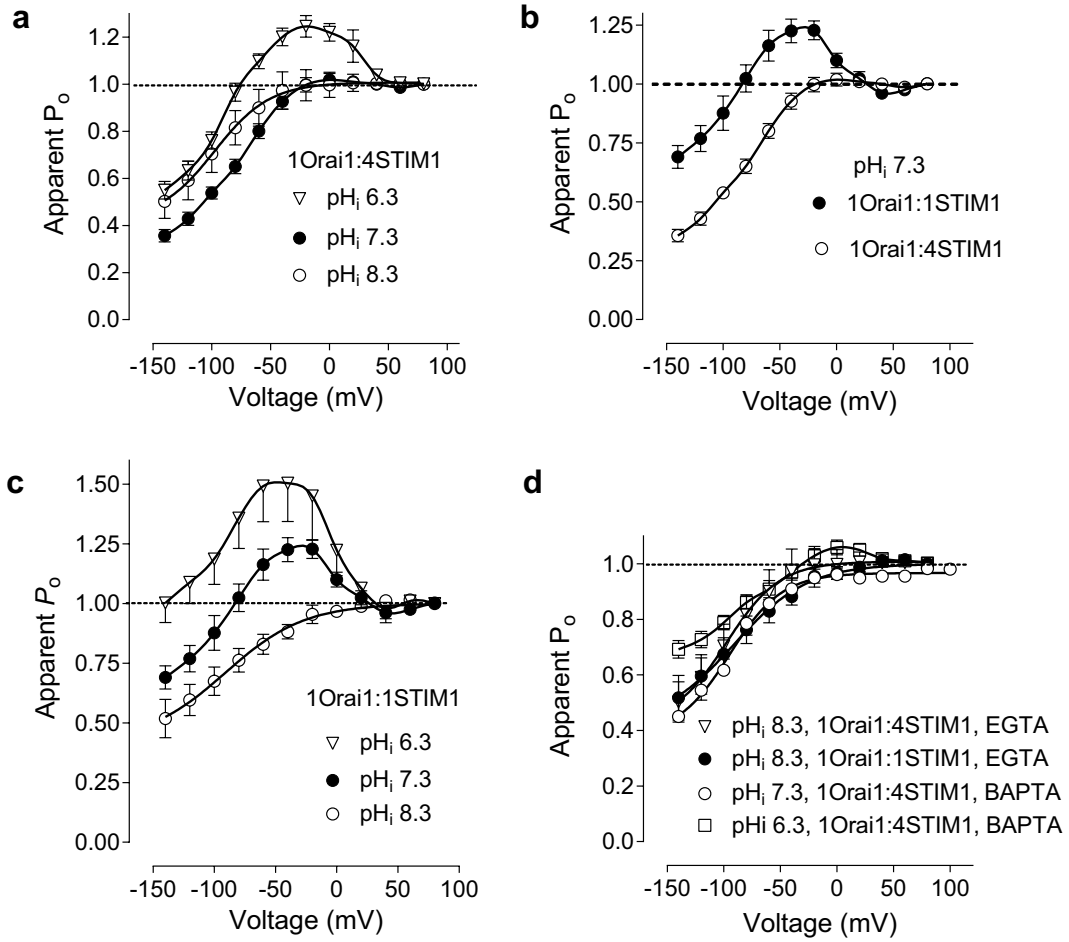

Figure 2. The dependence of WT I_{CRAC} apparent P_o on pH_i. (**a**) Apparent P_o curves were obtained using tail currents as described in Methods. HEK293T cells were transfected with Orai1 and STIM1 at 1:4 ratio and I_{CRAC} was recorded at pH_i 6.3 (clear triangles, n = 6), 7.3 (filled circles, n = 16) and 8.3 (clear circles, n = 7). (**b**) The comparison of P_o curves obtained at Orai1:STIM1 transfection ratios 1:1 (filled circles, n = 9) and 1:4 (clear circles, n = 16) at pH_i 7.3. (**c**) Apparent P_o curves obtained from tail currents recorded in cells transfected with Orai1 and STIM1 at 1:1 ratio. I_{CRAC} was recorded at pH_i 6.3 (clear triangles, n = 4), 7.3 (filled circles, n = 9) and 8.3 (clear circles, n = 8). Data on panels (**a**,**b** and **c**) were obtained with pipette solution containing EGTA. (**d**) The effect of BAPTA on P_o. Apparent P_o curves were obtained using cells transfected with Orai1 and STIM1 at 1:4 transfection ratio and BAPTA in the pipette solution at pH_i 7.3 (clear circles) and 6.3 (clear squares). For comparison, P_o curves obtained using cells transfected with Orai1 and STIM1 at either 1:4 (clear triangles) or 1:1 (filled circles) transfection ratio and EGTA in the pipette solution at pH_i 8.3 are shown.

To investigate whether intracellular Ca^{2+} buffer contributes to the dependence of I_{CRAC} on pH_i, we used extracellular application of 30 mM NH_4Cl, which is known to alkalinise pH_i[13,21]. Application of NH_4Cl to the bath, when EGTA was used as Ca^{2+} buffer in the pipette solution, drastically increased the I_{CRAC} amplitude (Fig. 3a,b) and caused inhibition of both I_{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_4Cl when BAPTA was used in the pipette solution instead of EGTA, had very little effect on I_{CRAC} amplitude (Fig. 3a,b).

Is there any overlap between mechanisms regulating I_{CRAC} dependence on pH_o and pH_i? Previous investigations have shown that the amplitude of native I_{CRAC} in different cell types and I_{CRAC} mediated by heterologously expressed Orai1 and STIM1 strongly depends on extracellular pH[8–10,12]. Superficially, the dependence of I_{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_{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_{CRAC} re-activation at negative potentials (Fig. 2b), and if raising pH_o results in a rise of pH_i, 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_{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.

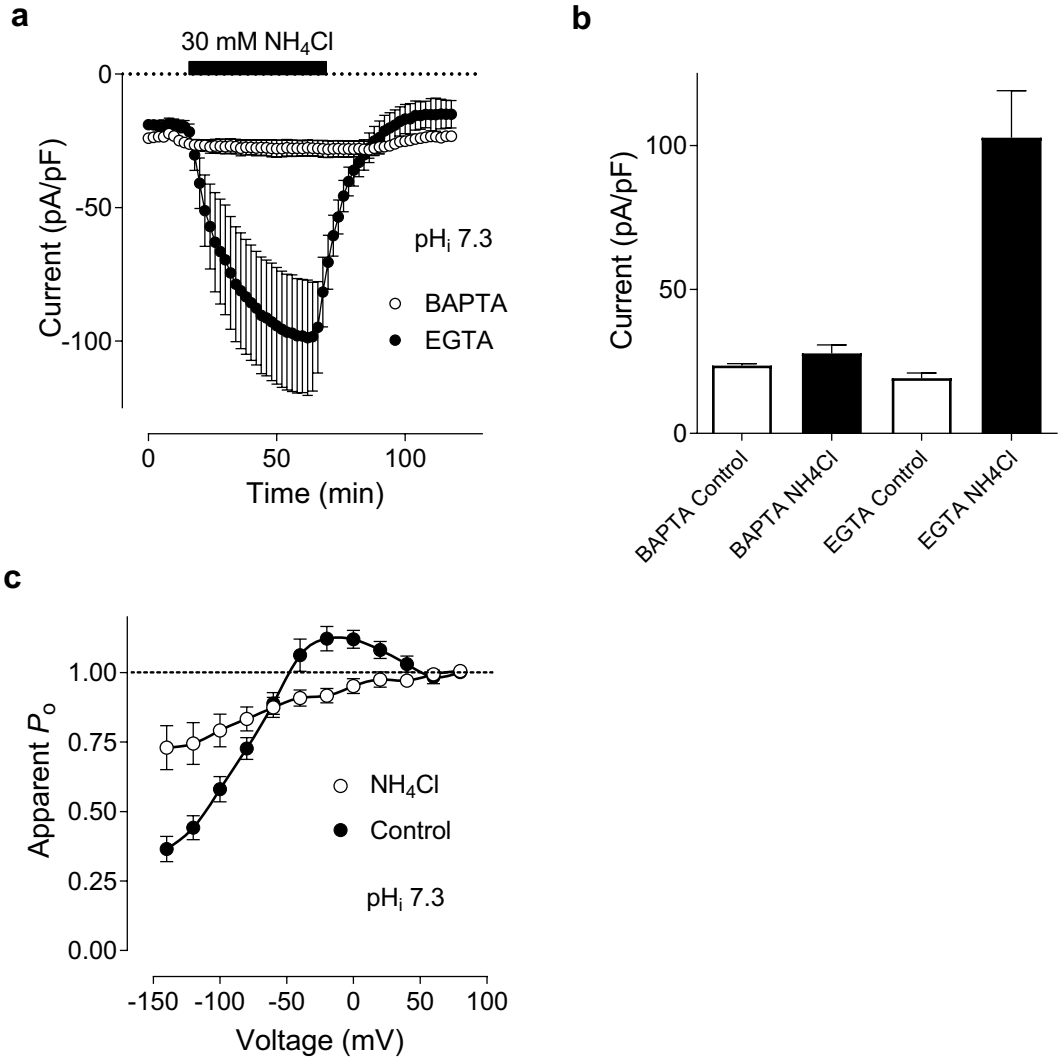

Figure 3. I_{CRAC} potentiation by intracellular alkalinisation depends on intracellular Ca^{2+} buffer. (**a**,**b**) The effect of 30 mM NH_4Cl application to the bath on the amplitude of I_{CRAC}. Each point on panel **a** represents I_{CRAC} amplitude at −100 mV obtained from 100 ms voltage ramps between −120 and 120 mV, applied every 2 s. WT I_{CRAC} was recorded in cells transfected with Orai1 and STIM1 at 1:4 ratio using either 10 mM BAPTA (clear circles) or 10 mM EGTA (filled circles) in the pipette solution (n = 5 for each condition), pH_i 7.3. (**c**) Apparent P_o curves obtained before (filled circles) and after (clear circles) application of NH_4Cl to the bath (n = 5).

One of the main residues responsible for I_{CRAC} dependence on pH_o is Glu 106 in the Orai1 pore[9]. Could pH_i affect protonation of Glu 106 in the Orai1 pore? Despite the observation that I_{CRAC} kinetics is unaffected by pH_o, it is possible that I_{CRAC} amplitude dependence on pH_i and pH_o 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[9]. Firstly, it is less selective for Ca^{2+} and supports a significant Na^+ conductance. Secondly, while E106D-mediated I_{CRAC} exhibits strong inactivation at negative potentials that looks similar to FCDI of WT I_{CRAC} (Fig. 5a, *cf.* Fig. 1b), it has a different underlying mechanism. The inactivation of E106D-mediated I_{CRAC} during steps to negative potentials is caused by Ca^{2+} block of Na^+ permeation through the pore; it does not require interaction with ID_{STIM}, and it is not affected by BAPTA or Orai1:STIM1 transfection ratios[9]. Finally, and importantly for this investigation, the Ca^{2+} dependent block of Na^+ permeation through the E106D pore is strongly pH_o dependent, whereas the peak amplitude of I_{CRAC} mediated by E106D Orai1 is not influenced by pH_o[9]. Changing the pipette solution pH revealed that the amplitude of E106D-mediated I_{CRAC} was pH_i-dependent – the current was strongly inhibited by pH_i 6.3 and enhanced by pH_i 8.3, similarly to WT I_{CRAC} (Fig. 5c, *cf.* Fig. 1a). E106D-mediated I_{CRAC} recorded in the absence of Na^+ in the bath solution, when Ca^{2+} was the only permeating cation, also showed pH_i dependence of the amplitude similar to that of WT I_{CRAC} (Fig. 5d). However, the kinetics and the extent of Ca^{2+} dependent block of Na^+ permeation through E106D Orai1 was not appreciably affected by pH_i (Fig. 5b, *cf.* Figs 5a and 1d). If Asp 106 could be protonated from the intracellular side at low pH_i, one would expect the changes in E106D-mediated I_{CRAC} to be similar to those induced by low pH_o[9],

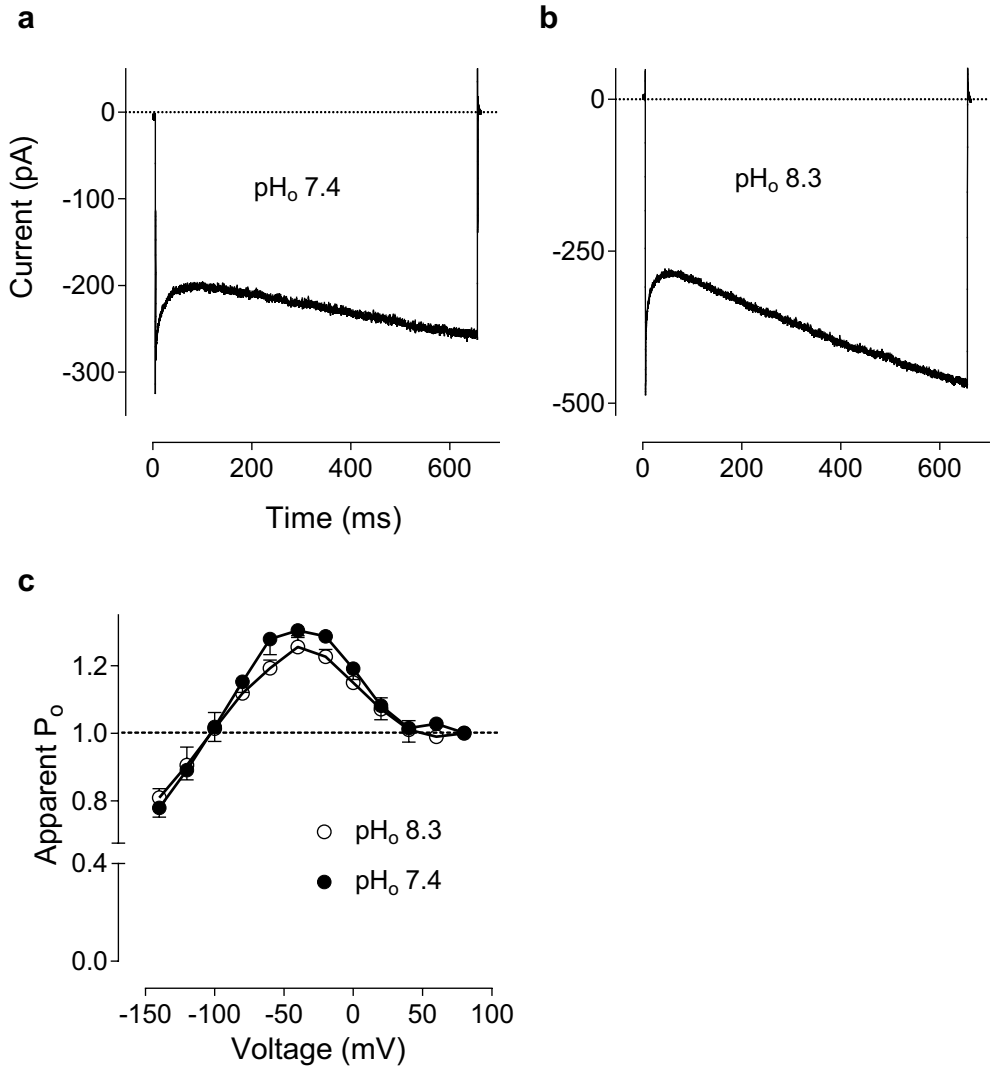

Figure 4. Extracellular alkalinisation has no effect on I_{CRAC} kinetics. (**a**,**b**) Examples of I_{CRAC} traces recorded in response to −120 mV steps in the bath solution of pH_o 7.4 and after changing pH_o to 8.3, correspondingly. (**c**) Apparent P_o curves at pH_o 7.4 (filled circles) and after 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.

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)[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[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)[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: 4 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_i 8.3 reduced the re-activation component (Fig. 7a), as it did in WT I_{CRAC} (Fig. 2a), pH_i 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_i 6.3 as the amplitude of the current was too small for a reliable extraction of the data.

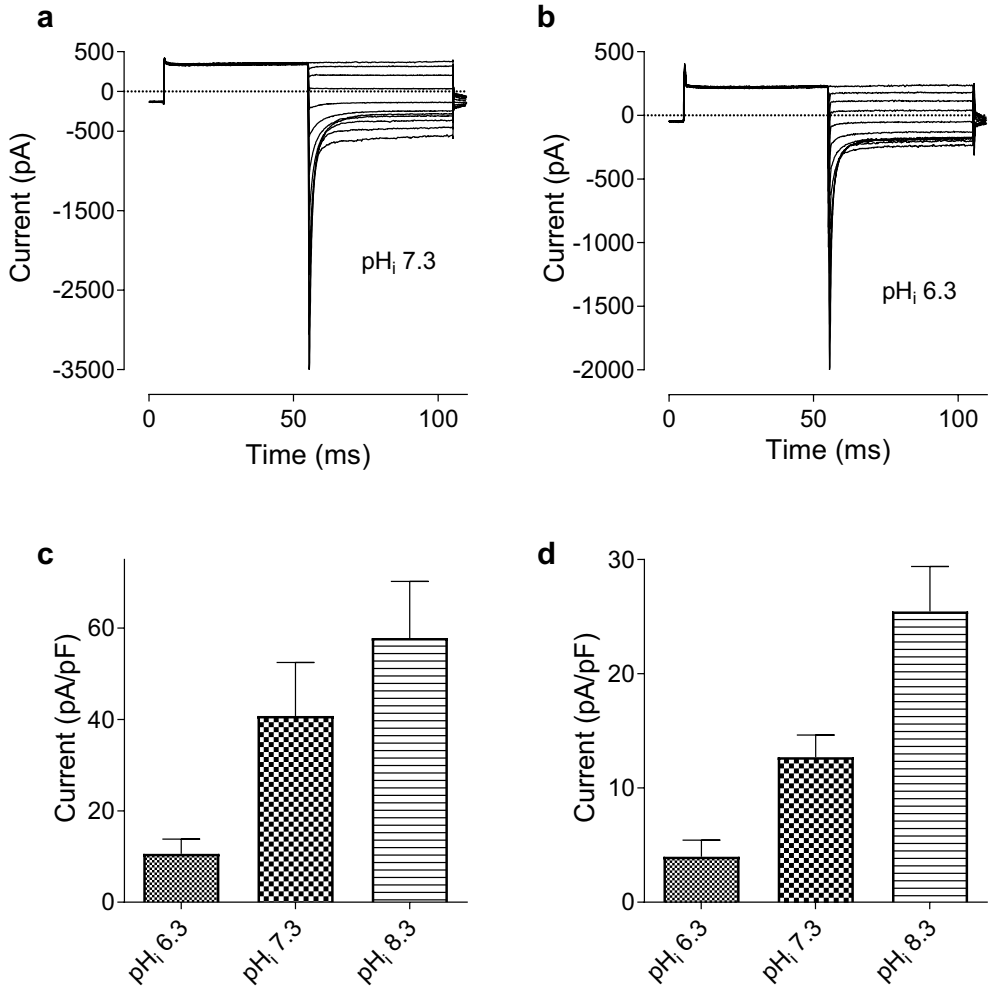

Figure 5. pH$_i$ dependence of E106D-mediated I_{CRAC}. (**a**,**b**) E106D Orai1 current traces were recorded in response to voltage steps ranging from −120 mV to 80 mV, in 20 mV increments, after a pre-pulse to 80 mV at pH$_i$ 7.3, and pH$_i$ 6.3, correspondingly. (**c**,**d**) The dependence of E106D Orai1 mediated I_{CRAC} amplitude on pH$_i$ in the control bath solution (**c**), and in a bath solution with 140 mM NaCl replaced with 140 mM NMDGCl (**d**). HEK293T cells were transfected with E106D Orai1 and STIM1 plasmids at 1:4 molar ratio.

I_{CRAC} 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]. The higher the expression of Orai1, relative to STIM1, the stronger the potentiation[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

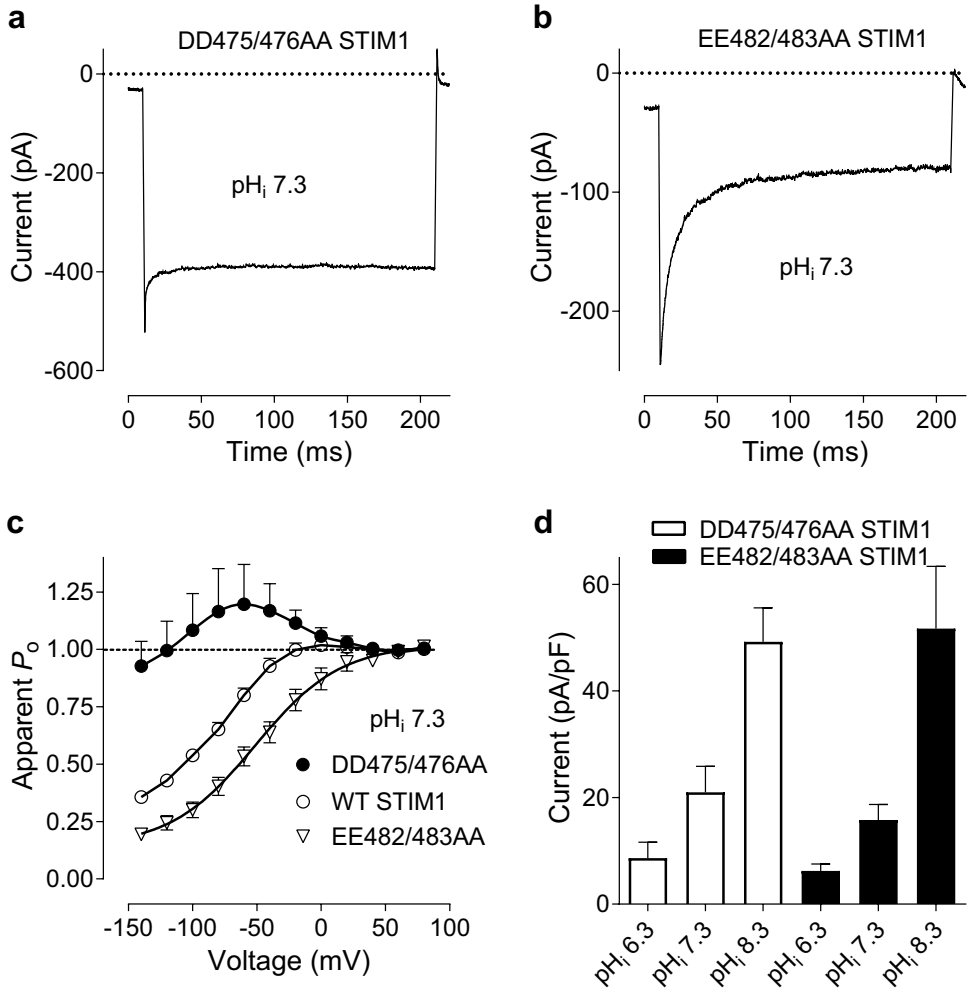

Figure 6. The dependence of I_{CRAC} amplitude, mediated by Orai1 and DD475/6AA and EE482/3AA STIM1 mutants, on pH_i. (**a**,**b**) Examples of I_{CRAC} traces recorded in response to 200 ms voltage steps from 0 mV to −120 mV in cells transfected with Orai1 and either DD475/6AA STIM1 (**a**) or EE482/3AA STIM1 (**b**) at pH_i 7.3. (**c**) Apparent P_o curves obtained at pH_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_{STIM} mutants I_{CRAC} amplitude was measured at −100 mV from the responses to 100 ms voltage ramps from −120 to 120 mV, at indicated pH_i. HEK293T cells were transfected with Orai1 and DD475/6AA STIM1 or EE482/3AA STIM1 plasmids at 1:4 ratio.

presence of EGTA in the pipette solution is a result of pH dependence of the Ca^{2+} buffering properties of EGTA, not the CRAC channel itself; (iii) Glutamate 106 in the selectivity centre of Orai1 pore, which mediates I_{CRAC} dependence on pH_o, does not contribute to I_{CRAC} dependence on pH_i; (iv) negatively charged residues in ID_{STIM} domain play a role in pH_i regulation of CRAC channel gating kinetics but not the amplitude of I_{CRAC}. These data suggest that several mechanisms contribute to I_{CRAC} regulation by pH_i.

It has been shown previously that increasing the amounts of Orai1 relative to STIM1 results in a smaller I_{CRAC} that exhibits re-activation at negative potentials which masks FCDI[15,16]. The results presented here show that intracellular acidification has an effect on I_{CRAC} similar to that of increasing the relative amounts of Orai1 (or decreasing the relative amounts of STIM1). Comparable changes in I_{CRAC} kinetics and amplitude caused by intracellular acidification and increased Orai1:STIM1 ratio suggest that low pH_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 Orai1and STIM1 and inhibits I_{CRAC}[13]. In the study of Mancarella *et al.* (2012) the effect of hypoxia on I_{CRAC} could be mimicked by application of extracellular propionate, which lowers pH_i, and reversed by application of NH_4Cl, which raises pH_i[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[13]. These results suggested that pH_i affects STIM1/Orai1 functional coupling leading to channel opening, but not the interactions that trap STIM1 and Orai1 in puncta[13]. The pH dependent changes in I_{CRAC} kinetics reported here also point to the conclusion that intracellular acidification disrupts STIM1/Orai1 functional interactions.

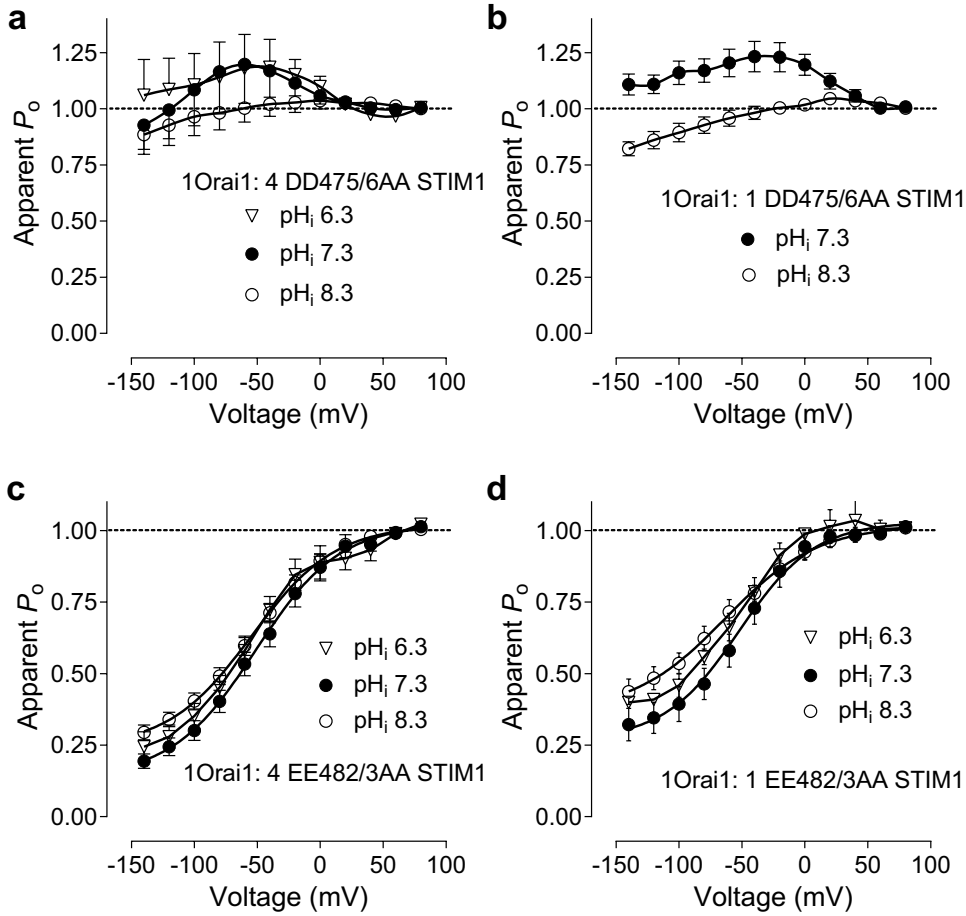

Figure 7. pH$_i$ has no effects on the apparent P_o of I_{CRAC} mediated by Orai1 and DD475/6AA and EE482/3AA STIM1. (**a**,**b**) Apparent P_o curves obtained using cells transfected with Orai1 and DD475/6AA STIM1 at either 1:4 (**a**) or 1:1 (**b**) ratio and pH$_i$ 6.3 (clear triangles), 7.3 (filled circles) and 8.3 (clear circles). (**c**,**d**) Apparent P_o curves obtained using cells transfected with Orai1 and EE482/3AA STIM1 at either 1:4 (**a**) or 1:1 (**b**) ratio and pH$_i$ 6.3 (clear triangles), 7.3 (filled circles) and 8.3 (clear circles).

Inhibition of I_{CRAC} by low pH$_i$ has been demonstrated previously in several publications[8,10,12]. They all agree that I_{CRAC}, both endogenous and mediated by ectopically expressed Orai1 and STIM1, is inhibited by approximately 70–90% at pH$_i$ of around 6, compared to pH$_i$ 7.3[8,10,12]. In contrast, the effects of alkalinisation of pH$_i$ above 7.3 on I_{CRAC} are inconsistent between different studies[8,10,12]. 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 I_{CRAC} amplitude at higher pH$_i$, whereas studies that used EGTA reported a significant potentiation of I_{CRAC} amplitude by alkalinisation[8,10,12]. Calculations using Maxchelator (http://maxchelator.stanford.edu/) 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 1.28×10^{-7} M at pH 7.3 to 1.4×10^{-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 I_{CRAC} amplitude in response to NH_4Cl 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 I_{CRAC} pH$_i$ dependence in the presence of EGTA, particularly, when pH$_i$ rises above 7.5. The increase in I_{CRAC} amplitude at alkaline pH$_i$ is likely to be due to stronger and faster Ca^{2+} binding by EGTA, rather than increase in pH$_i$ *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[23,24]. This is supported by the observations that intracellular alkalinisation induced by application of NH_4Cl 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[21,25]. Therefore, results obtained using EGTA, rather than BAPTA, may have more physiological relevance. Much bigger amplitude of I_{CRAC} activated by IP_3 in the presence of BATPA in the pipette solution, compared to EGTA, was noticed very early on 19. However, the reason for this difference remains poorly understood.

The only residue that has been implicated in I_{CRAC} dependence on pH$_i$ so far is His 155 in Orai1[10]. H155F mutation in Orai1 was shown to abolish the increase of I_{CRAC} amplitude in response to intracellular alkalinisation, but I_{CRAC} mediated by H155F-Orai1 was still inhibited by about 60% at low pH$_i$[10], which implies that His 155 is

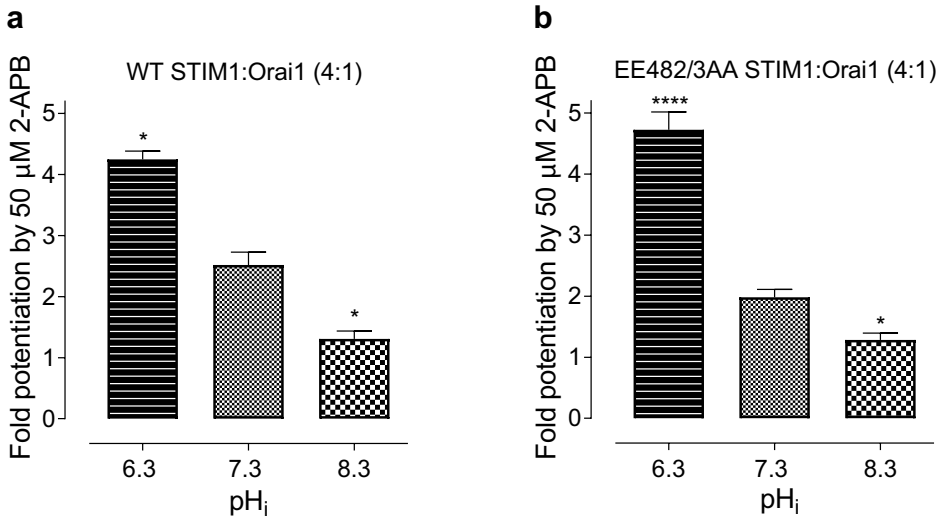

Figure 8. The pH_i dependence of I_{CRAC} potentiation by 2-APB. The Y-axis represents the ratio between the amplitudes of I_{CRAC} recorded immediately after and before application of 50 µM 2-APB to the bath. The I_{CRAC} amplitude was measured at −100 mV from the responses to 100 ms voltage ramps from −120 to 120 mV, applied every 2 seconds. HEK 293 T cells were transfected with WT STIM1 and Orai1 (**a**) or EE482/3AA STIM1and Orai1(**b**) at 4:1 ratio. pH of the pipette solution is indicated below the bars.

unlikely to be the only site that mediates I_{CRAC} regulation by pH_i. Data presented in this work indicate that Glut 106 in the Orai1 selectivity centre, which can be protonated from the extracellular side[9], 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 pH_i sensor of CRAC channel[18]. 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[18], *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, EE482/483AA-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 expression of the mutant STIM1, compared to Orai1. If the expression levels of STIM1 are very high, moderate changes in the affinity of STIM1 biding to 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}[16]. 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) CO_2 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 serum[9,16]. 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[9,16]. 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[9,16]. The control bath solution contained 140 mM NaCl, 4 mM CsCl, 10 mM $CaCl_2$, 2 mM $MgCl_2$ and 10 mM HEPES adjusted to pH 7.4 with NaOH. Depletion of intracellular Ca^{2+} stores was achieved using 20 μM Ins(3,4,5)*P*3 (Sigma) added to an internal solution containing 130 mM caesium glutamate, 10 mM CsCl, 5 mM $MgCl_2$, 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 I_{CRAC}, or after its inhibition with 10 µM La^{3+} were used for leakage subtraction.

Data analysis. To obtain apparent (relative) open probability (P_o) 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 s 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 I_{CRAC} – 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:

$$P_o(V) = P_{min} + (1 - P_{min})/(1 + \exp((V_{1/2} - V)/k)) \quad (1)$$

where P_{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. *The Journal of Physiology* **537**, 993–998 (2001).
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. Swietach, P., Vaughan-Jones, R. D., Harris, A. L. & Hulikova, A. The chemistry, physiology and pathology of pH in cancer. *Philosophical Transactions of the Royal Society B: Biological Sciences* **369** (2014).
6. Taylor, S. *et al*. 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 Ca^{2+}: Ionic Allies in Tumor Progression? *Physiology* **26**, 252–265 (2011).
8. Beck, A., Fleig, A., Penner, R. & Peinelt, C. Regulation of endogenous and heterologous Ca^{2+} release-activated Ca^{2+} 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 Ca^{2+}-dependent inactivation and pH dependence of Ca^{2+} release-activated Ca^{2+} (CRAC) current. *Biochem J* **441**, 743–753 (2012).
10. Tsujikawa, H. *et al*. Identification of key amino acid residues responsible for internal and external pH sensitivity of Orai1/STIM1 channels. *Sci Rep* **5**, 16747 (2015).
11. Malayev, A. & Nelson, D. J. Extracellular pH modulates the Ca^{2+} current activated by depletion of intracellular Ca^{2+} stores in human macrophages. *J Membr Biol* **146**, 101–111 (1995).
12. Csutora, P. *et al*. Activation mechanism for CRAC current and store-operated Ca^{2+} entry - Calcium influx factor and Ca^{2+}-independent phospholipase A(2)beta-mediated pathway. *Journal of Biological Chemistry* **281**, 34926–34935 (2006).
13. Mancarella, S. *et al*. Hypoxia-induced Acidosis Uncouples the STIM-Orai Calcium Signaling Complex. *Journal of Biological Chemistry* **286**, 44788–44798 (2011).
14. Frischauf, I. *et al*. Cooperativeness of Orai cytosolic domains tunes subtype-specific gating. *J Biol Chem* **286**, 8577–8584 (2011).
15. Hoover, P. J. & Lewis, R. S. Stoichiometric requirements for trapping and gating of Ca Ca^{2+}release-activated Ca Ca^{2+} (CRAC) channels by stromal interaction molecule 1 (STIM1). *Proc Natl Acad Sci USA* **108**, 13299–13304 (2011).
16. Scrimgeour, N., Litjens, T., Ma, L., Barritt, G. J. & Rychkov, G. Y. Properties of Orai1 mediated store-operated current depend on the expression levels of STIM1 and Orai1 proteins. *J Physiol* **587**, 2903–2918 (2009).
17. Mullins, F. M. & Lewis, R. S. The inactivation domain of STIM1 is functionally coupled with the Orai1 pore to enable Ca^{2+} dependent inactivation. *J Gen Physiol* **147**, 153–164 (2016).
18. Mullins, F. M., Park, C. Y., Dolmetsch, R. E. & Lewis, R. S. STIM1 and calmodulin interact with Orai1 to induce Ca^{2+}-dependent inactivation of CRAC channels. *Proc Natl Acad Sci USA* **106**, 15495–15500 (2009).
19. Hoth, M. & Penner, R. Calcium release-activated calcium current in rat mast cells. *J. Physiol.* **465**, 359–386 (1993).
20. Zweifach, A. & Lewis, R. S. Rapid inactivation of depletion-activated calcium current (I_{CRAC}) due to local calcium feedback. *J. Gen. Physiol.* **105**, 209–226 (1995).
21. Marumo, M. & Wakabayashi, I. Intracellular alkalinization augments capacitative Ca^{2+}entry in platelets. *Thromb Haemost* **90**, 1121–1127 (2003).
22. Prakriya, M. & Lewis, R. S. Potentiation and inhibition of Ca^{2+} release-activated Ca^{2+} channels by 2-aminoethyldiphenyl borate (2-APB) occurs independently of IP_3 receptors. *J. Physiol.-London* **536**, 3–19 (2001).
23. Schwaller, B. Cytosolic Ca^{2+} buffers. *Cold Spring Harb Perspect Biol* **2**, a004051 (2010).
24. 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).
25. Nitschke, R. *et al*. The effect of intracellular pH on cytosolic Ca^{2+} in HT29 cells. *Pflugers Archiv: European journal of physiology* **433**, 98–108 (1996).

Acknowledgements
We would like to thank Prof Richard Lewis, Stanford University, for providing STIM1 double mutants. This work was supported by the Australian Research Council, Discovery Project 140100259.

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
Competing Interests: The authors declare that they have no competing interests.

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.