Activation of store-operated $I_{\text{CRAC}}$ by hydrogen peroxide

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Reactive oxygen species (ROS) such as hydrogen peroxide ($H_2O_2$) play a role in both innate immunity as well as cellular injury. $H_2O_2$ induces changes in intracellular calcium ($[Ca^{2+}]_i$) in many cell types and this seems to be at least partially mediated by transient receptor potential melastatin 2 (TRPM2) in cells that express this channel. Here we show that low concentrations of $H_2O_2$ induce the activation of the $Ca^{2+}$-release activated $Ca^{2+}$ current $I_{\text{CRAC}}$. This effect is not mediated by direct CRAC channel activation, since $H_2O_2$ does not activate heterologously expressed CRAC channels independently of stromal interaction molecule (STIM). Instead, $I_{\text{CRAC}}$ activation is partially mediated by store depletion through activation of inositol 1,4,5 trisphosphate receptors (IP$_3$R), since pharmacological inhibition of IP$_3$ receptors by heparin or molecular knock-out of all IP$_3$ receptors in DT40 B cells strongly reduce $H_2O_2$-induced $I_{\text{CRAC}}$. The remainder of $H_2O_2$-induced $I_{\text{CRAC}}$ activation is likely mediated by IP$_3$R-independent store-depletion. Our data suggest that $H_2O_2$ can activate $Ca^{2+}$ entry through TRPM2 as well as store-operated CRAC channels, thereby adding a new facet to ROS-induced $Ca^{2+}$ signaling.

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1. Introduction

Reactive oxygen species (ROS) are a group of molecules and ions with the potential of causing cellular damage due to their highly reactive characteristics. Hydrogen peroxide ($H_2O_2$), an oxidizing agent and member of ROS, is often used in experimental models of oxidative stress, although accumulating evidence indicates that $H_2O_2$ may also function as an important signaling molecule in diverse cellular processes such as cell development, proliferation, signal transduction and protein regulation [1]. Thus, ROS and intracellular $Ca^{2+}$ have shown interdependent relations in numerous processes [2], thereby linking ROS to one of the most diverse second messenger systems in the cell. $H_2O_2$ is constantly produced in the cell as a by-product of aerobic metabolism in the mitochondria. To prevent toxic ROS overload, several enzymatic mechanisms will catalyze the conversion of $H_2O_2$ to water and oxygen, including peroxisomal catalase and cytosolic peroxiredoxins and glutathione peroxidase. For a long time, $H_2O_2$ was believed to freely cross membranes, but more recent evidence suggests that the membrane permeability is regulated by the composition of the membrane as well as diffusion facilitation through aquaporins [3].

The broad effects of $H_2O_2$ on enzymes, growth factors, transcription factors and ion channels are believed to mainly occur through redox modification of reactive thiol groups in cysteine residues [4]. Although various ion channels are modulated by ROS [4,5], the only known ion channel that can be gated through actions of $H_2O_2$ is the $Ca^{2+}$ permeable non-selective cation channel TRPM2 (transient–receptor potential melastatin-2) [6–8]. The channel’s primary agonist is adenosine diphosphoribose (ADPR) [9,10]. Intracellular $Ca^{2+}$, $H_2O_2$ or related nucleotides such as cyclic adenosine diphosphoribose (cADPR) and nicotinic acid adenine dinucleotide phosphate (NAADP) have limited if any direct gating activities [7,11], although they can synergize with ADPR to activate TRPM2 currents at lower ADPR concentrations [7,12]. For $H_2O_2$-mediated activation of TRPM2 both ADPR-dependent and independent mechanisms have been suggested [8,13]. Additional channel targets of $H_2O_2$ include the ryanodine receptor (RyR), which functions as a $Ca^{2+}$-release channel in muscle cells and neurons [14] and an outwardly rectifying cation current, $I_{\text{OC}}$, in murine cortical neurons, presumably involving TRPM7 channels [15]. Additionally, $H_2O_2$ at millimolar concentration can induce a sustained nonselective cation current, $I_{\text{INAC}}$, independent of ion channels [16].

The major route of $Ca^{2+}$ entry in nonexcitable cells is via store-operated channels (SOC). The best characterized SOC is the $Ca^{2+}$ release-activated $Ca^{2+}$ channel (CRAC), a highly selective low-conductance $Ca^{2+}$ channel, which is activated by depletion of internal $Ca^{2+}$ stores [17]. Molecularly, this involves stromal interaction molecule 1 (STIM1), which senses ER $Ca^{2+}$ levels [18,19] and upon store depletion activates the plasma membrane channel CRACM1 (also called Orai1) [20–22]. Store-operated $Ca^{2+}$ entry (SOCE) is a widely expressed mechanism in many cells that respond to ROS, however, little is known about effects of ROS on the store-
operated CRAC current (I_{CRAC}). We set out to assess possible effects of ROS on I_{CRAC} using calcium imaging and whole-cell electrophysiology. We report that extracellular application or intracellular perfusion of H$_2$O$_2$ at micromolar concentrations activates I_{CRAC} in several native cell lines independent of the presence or absence of TRPM2-like currents.

2. Methods

2.1. Cell culture

Cells were incubated at 37 °C with 5% CO$_2$ in the appropriate cell media. Tetracycline-inducible HEK293 TRPM2-expressing cells were cultured in DMEM with 10% fetal bovine serum (FBS) supplemented with basicidin (5 µg/ml, Invitrogen) and zeocin (0.4 mg/ml, Invitrogen) [9], RBL-2H3 cells in DMEM with 10% FBS, Jurkat T-lymphocytes in RPMI 1640 with 10% FBS, HEK293 CRACM1-overexpressing cells in DMEM with 10% FBS [23], and DT40 B-lymphocytes in RPMI 1640 with 10% FBS supplemented with 5% chicken serum and 2 mM l-glutamine. For induction of TRPM2 expression, HEK293 cells were resuspended in medium containing 1 µg/ml tetracycline (Invitrogen) 4–8 h before experiments.

2.2. Solutions and chemicals

For fluorescence and patch-clamp measurements cells were kept in standard extracellular saline solution (in mM): 140 NaCl, 2.8 KCl, 2 MgCl$_2$, 11 glucose, 10 HEPES–NaOH. CaCl$_2$ concentration was 1 mM in calcium imaging experiments and 10 mM in patch-clamp experiments unless otherwise stated. Neutral pH was between 7.2 and 7.3, adjusted with NaOH and osmolarity was 300 mOsm. For the TRPM2 inhibition panel the divalent and trivalent cations (Ba$^{2+}$, Cd$^{2+}$, Cu$^{2+}$, Ni$^{2+}$, Sr$^{2+}$, Zn$^{2+}$, and La$^{3+}$) were added as chloride salts into the standard extracellular solution at 1 mM. In some experiments 1 µM LaCl$_3$ was included in the extracellular solution. H$_2$O$_2$ (30% stock solution) was added in various concentrations to TRPM2 from other Ca$^{2+}$ entry pathways, including I_{CRAC}. The low-resolution temporal development of currents for a given potential was extracted from the leak-corrected individual ramp currents by measuring current amplitudes at voltages of −80 mV and +80 mV, unless otherwise stated.

2.4. Single channel measurements

TRPM2 single channel recordings in Jurkat T cells were performed in the whole-cell configuration. A ramp from −100 mV to +100 mV over 11 s was applied continuously. Currents were filtered at 50 Hz. Linear fits were performed from −100 mV to 0 mV in Igor Pro (WaveMetrics, Oregon, USA) to determine the single channel conductance $b$ according to a line function $a + bx$.

2.5. Fluorescence measurements

For measurement of cytosolic Ca$^{2+}$ concentration cells were loaded with 5 µM Fura-2 AM (acetoxyxymethylene, Molecular Probes) for 30 min in 37 °C, washed and kept in external solution. Experiments were performed with an Olympus BX2 fluorescence microscope equipped with a dual excitation fluorometric imaging system (TILL-Photonics). Data acquisition and computation was controlled by TILLvision software. Dye-loaded cells were excited by wavelengths of 340 nm and 380 nm for 20 ms each, produced by a monochromator (Polychrome IV). The fluorescence emission of several single cell bodies was simultaneously recorded with a video camera (TILL-Photonics Imago) with an optical 440 nm long-pass filter. The signals were sampled at 0.5 Hz and computed into relative ratio units of the fluorescence intensity at the different wavelengths (340/380 nm).

2.6. Subcloning and overexpression

Full length human STIM1 was subcloned as described earlier [24]. For electrophysiological analysis, STIM1 proteins were overexpressed in HEK293 cells stably expressing CRACM1 [25] using lipofectamine 2000 (Invitrogen) and the GFP expressing cells were selected by fluorescence. Experiments were performed 24–48 h post-transfection.

2.7. Data analysis

Data was analyzed with FitMaster (HEKA, Lambrecht, Germany) and Igor Pro (WaveMetrics, Oregon, USA). Where applicable, statistical errors of averaged data are given as means ± SEM with $n$ determinations. Single ramps were plotted as current–voltage relationships. Currents were normalized to cell size in pF. Activation time and maximum current plateau phase were determined and corrected before each voltage ramp. The low-resolution temporal development of currents for a given potential was extracted from the leak-corrected individual ramp currents by measuring current amplitudes at voltages of −80 mV and +80 mV, unless otherwise stated.

3. Results

3.1. TRPM2 currents are not significantly affected by divalent or trivalent ions

H$_2$O$_2$ is a well-known activator of TRPM2 currents [6–8]. We pursued two strategies to differentiate H$_2$O$_2$-induced Ca$^{2+}$ influx through TRPM2 from other Ca$^{2+}$ entry pathways, including I_{CRAC}. The first strategy involved the use of cell lines that differentially
express ICRAC and TRPM2 and the second was based on differential pharmacological inhibition. Known blockers of TRPM2, such as 2-amino-ethoxydiphenyl borate (2-APB), flufenamic acid or clotrimazole [26,27] also interfere with store-operated calcium entry [28–30]. Lanthanum (La3+), however, potently blocks ICRAC [31], but reportedly does not inhibit TRPM2 activity [32]. We tested various divalent cations and La3+ for inhibitory actions on TRPM2 currents in whole-cell patch-clamp experiments in HEK293 cells stably expressing TRPM2 under a tetracycline-inducible promoter. Cells were kept in a standard NaCl-based extracellular solution supplemented with 500 μM ADPR (see Section 2). Within seconds of whole-cell break-in, large currents developed (Fig. 1A) with the typical linear current–voltage (I/V) relationship of TRPM2 (Fig. 1B), reaching several nA at ~80 mV. In order to measure the inhibition of TRPM2, currents were allowed to reach the plateau phase before application of 1 mM of various divalent cations or La3+ at 60 s. When applying Ba2+, Cu2+, Ni2+, Cd2+, Sr2+, or Co2+ current reduction was less than 5% and Zn2+ and La3+ reduced currents by just 5–7% (Fig. 1C). In each case, the current reduction was reversible as seen by the increase in current following removal of the cations at 120 s. This demonstrates that TRPM2 is not markedly affected by divalent ions or La3+, in marked contrast to many other ion channels, including ICRAC [31].

### 3.2. H2O2 causes Ca2+ release and Ca2+ influx independent of TRPM2

Using Fura-2 calcium imaging, we assessed Ca2+-permeable ion channels as candidates for H2O2-mediated Ca2+ signals in various cell types that express ICRAC with or without TRPM2. Jurkat T lymphocytes express both ICRAC and TRPM2 natively [11,33], whereas RBL-2H3 (Fig. S1A; [34]) and wild-type (WT) HEK293 express only ICRAC [9,21]. In the latter cell type, we additionally overexpressed TRPM2 heterologously. Cells were bathed in standard external NaCl-based bath solution with 1 mM Ca2+ and then exposed for 3 min to 100 μM H2O2 and 200 μM MLaCl3, which has negligible inhibitory effects on TRPM2 (Fig. 1B), reaching several nA at ~80 mV. Inward currents were extracted at a holding potential of 0 mV. Inward currents were normalized to the data point prior to cation application. The bar indicates application of 1 mM of each cation (n=5 for each condition), displayed is the Ba2+ and La3+ trace. Currents were measured with a voltage ramp from −100 to +100 mV over 50 ms at 0.5 Hz intervals from a holding potential of 0 mV. Inward currents were extracted at ~80 mV, averaged and plotted versus time. (B) Representative current–voltage (I/V) relationship of ADPR-activated TRPM2 current in HEK293 cells at t=60 s and upon application of 1 mM La3+ at t=120 s. (C) Degree of inhibition of TRPM2 by 1 mM of various di- and trivalent ions. Values represent mean (±SEM) over the 60 s period of application.

![Image](388x354 to 572x784)

Fig. 1. Inhibition of TRPM2 current by various divalent and trivalent cations. (A) Average TRPM2 currents in tetracycline-induced HEK293 cells (1 μg/ml, 4–8 h) perfused with Cs-glutamate-based pipette solution containing 500 μM ADPR with unbuffered internal Ca2+. Currents were normalized to the data point prior to cation application. The bar indicates application of 1 mM of each cation (n=5 for each condition), displayed is the Ba2+ and La3+ trace. Currents were measured with a voltage ramp from −100 to +100 mV over 50 ms at 0.5 Hz intervals from a holding potential of 0 mV. Inward currents were extracted at ~80 mV, averaged and plotted versus time. (B) Representative current–voltage (I/V) relationship of ADPR-activated TRPM2 current in HEK293 cells at t=60 s and upon application of 1 mM La3+ at t=120 s. (C) Degree of inhibition of TRPM2 by 1 mM of various di- and trivalent ions. Values represent mean (±SEM) over the 60 s period of application.

This was confirmed in HEK293 WT cells, where H2O2-induced Ca2+ release was followed by a Ca2+-signal upon Ca2+-readmission that could also be inhibited by 1 μM LaCl3 (Fig. 2B). It should be noted that in HEK293 WT cells 20 μM H2O2 was sufficient to evoke reliable Ca2+ responses, whereas in RBL-2H3 cells the H2O2 concentration had to be increased to 100 μM to reliably induce Ca2+ entry upon Ca2+-readmission. We next investigated Ca2+ release and Ca2+ influx in tetracycline-induced HEK293–TRPM2 cells. Applying 20 μM H2O2 resulted in Ca2+-release and substantial Ca2+ influx both in the absence and presence of 1 μM LaCl3 (Fig. 2C). This confirms that H2O2 can activate TRPM2 and that La3+ fails to block TRPM2.

We also investigated Jurkat T cells, which express TRPM2 and CRAC channels natively. Here, application of 20 μM H2O2 revealed Ca2+ release as well as Ca2+ influx when returning to 1 mM Ca2+ (Fig. 2D). To assess whether the Ca2+ entry upon readmission of Ca2+-involved TRPM2 activity, we performed the same experiment in the presence of 1 μM LaCl3, which has negligible inhibitory effects on TRPM2 (as seen in Figs. 1 and 2C). Again H2O2 induced a Ca2+ release, but Ca2+ entry was completely blocked (Fig. 2D), demonstrating that in Jurkat T cells the H2O2-mediated Ca2+ influx caused by 20 μM H2O2 was not related to TRPM2.

For all cell types investigated, the H2O2-induced Ca2+ signals trended back to the baseline in control experiments without Ca2+ readmission, but did not return entirely. The incomplete return to baseline may be due to an additional effect of H2O2 on the plasma membrane Ca2+ ATPase (PMCA). It has been previously reported that La3+ can cause an apparent prolongation of the release response due to a block of PMCA [35]. However, our data would imply that H2O2 is less potent than 1 μM La3+ in blocking PMCA. Our control data also provides evidence that Ca2+ entry through
Fig. 2. H2O2 induces Ca2+ response in RBL-2H3 cells, HEK293 cells and Jurkat T cells. (A) Averaged changes in [Ca2+]i measured as ratios of Fura-2 fluorescence excited at 340 and 380 nm in Fura-2 AM loaded RBL-2H3 cells in response to H2O2. Cells were kept in a saline solution with 1 mM CaCl2 in the absence (blue trace, n = 34) or presence of 1 μM LaCl3 (red trace, n = 35). Black bar indicates application of 100 μM H2O2 in nominally Ca2+ free saline solution. In controls H2O2 was omitted from the Ca2+ free saline solution (black trace, n = 57) and CaCl2 was omitted from the readmission solution (green trace, n = 42). Cells were loaded with 5 μM Fura-2 AM at 37°C for 30 min. Traces are representative of three independent experiments performed on different days. (B) Experimental protocol as in (A) but for Fura-2 AM loaded HEK293 WT cells in the absence (blue trace, n = 24) or presence of 1 μM LaCl3 (red trace, n = 6). Black bar indicates application of 20 μM H2O2 in Ca2+ free saline solution. In controls H2O2 was omitted from the Ca2+ free saline solution (black trace, n = 28) and CaCl2 was omitted from the readmission solution (green trace, n = 52). Traces are representative of three independent experiments performed on different days. (C) Experimental protocol as in (A) but for Fura-2 AM loaded HEK293 WT cells in the absence (blue trace, n = 16) or presence of 1 μM LaCl3 (red trace, n = 13). Black bar indicates application of 20 μM H2O2 in Ca2+ free saline solution. In controls H2O2 was omitted from the Ca2+ free saline solution (black trace, n = 9) and CaCl2 was omitted from the readmission solution (green trace, n = 28). (D) Experimental protocol as in (A) but for LaCl3 AM loaded Jurkat T cells in the absence (blue trace, n = 74) or presence of 1 μM LaCl3 (red trace, n = 126). Black bar indicates application of 20 μM H2O2 in nominally Ca2+ free saline solution. In controls H2O2 was omitted from the Ca2+ free saline solution (black trace, n = 49) and CaCl2 was omitted from the readmission solution (green trace, n = 15). Traces are representative of three independent experiments performed on different days. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

I_{CRAC} is more significant than the difference between the H2O2 and H2O2 + La3+ traces suggests. Together, these findings indicate that extracellular application of H2O2 at low micromolar concentrations can activate a Ca2+ influx pathway in Jurkat T cells, HEK293 WT cells and RBL-2H3 cells that is not related to TRPM2 activity.

3.3. H2O2 induces I_{CRAC} in RBL-2H3 and Jurkat T cells

To determine whether the H2O2-activated Ca2+ influx observed in Ca2+ imaging experiments was mediated by I_{CRAC}, we performed whole-cell patch-clamp experiments. We used standard experimental conditions optimized for measuring I_{CRAC}, i.e. NaCl-based extracellular solution with 10 mM CaCl2 and Cs-glutamate-based intracellular solution buffered to 150 mM free Ca2+ with Cs-BAPTA (see Section 2). In whole-cell patch-clamp experiments with RBL-2H3 cells, 10 mM CsCl was included in the bath solution to inhibit the inward-rectifier potassium current. We first tested for I_{CRAC} activation in RBL-2H3 cells by extracellular application of 40 μM H2O2 from a wide-tipped puffer pipette 1 min after break-in. As shown in Fig. 3A, H2O2 application activated an inward current in 12 out of 14 cells, reaching a plateau of $-2.5 \pm 0.3$ pA/pF at 600 s with a half-maximal activation time of 281 ± 1.2 s. No current developed in two cells. The current exhibited inward rectification that is typical for I_{CRAC} [17] as illustrated in a representative I/V relationship (Fig. 3B). When including 1 μM LaCl3 in the bath and application solution no inward current developed (Fig. 3A). In control experiments without H2O2 application, no significant inward currents developed (Fig. 3A).

Similar experiments were performed using Jurkat T cells. To effectively suppress spontaneous activation of I_{CRAC} in these cells, [Ca2+]i was buffered to 200 nM and [Mg2+]i was increased to 3 mM to suppress activation of endogenous TRPM7 currents. Application of 40 μM H2O2 did not activate inward currents consistently, however, 80 μM H2O2 activated an inward current in 5 of 5 cells. Currents reached a plateau of $-1.2$ pA/pF at 600 s with a half-
maximal activation time of $115 \pm 3.3$ s (Fig. 3C) and exhibited the typical inwardly rectifying I/V relationship for $I_{CRAC}$ (Fig. 3D). The $H_2O_2$-induced current could be completely blocked by 1 μM LaCl$_3$ in the bath solution (Fig. 3C). No significant inward current developed in control experiments when cells were not exposed to $H_2O_2$ (Fig. 3C). In summary, these data provide evidence for the ability of $H_2O_2$ to activate $I_{CRAC}$ in Jurkat T cells and in RBL-2H3 cells. It should be noted that none of the cells showed any sign of seal breakdown or increased leak current due to application of $H_2O_2$ at the concentrations used and neither did we observe development of any significant non-specific currents.

ROS are known to have a potential damaging effect on molecules such as DNA, proteins and lipids. We therefore investigated if $H_2O_2$, in addition to its activating qualities, exerted a negative effect on $I_{CRAC}$. We activated $I_{CRAC}$ by perfusing cells with 20 μM IP$_3$ and internal Ca$^{2+}$ buffered to 150 nM and then applied $H_2O_2$ after $I_{CRAC}$ had fully developed. In RBL-2H3 cells, we did not see any inhibiting effect of 40 μM $H_2O_2$ on $I_{CRAC}$ (Fig. 3E) and in Jurkat T cells, $I_{CRAC}$ currents were slightly reduced by about ~15% during application of 40 μM $H_2O_2$ (Fig. 3F). The relative lack of effect of $H_2O_2$ after $I_{CRAC}$ activation by IP$_3$ is further evidence that the current activated by $H_2O_2$ is exclusively $I_{CRAC}$ and not caused by other non-specific or previously unknown currents.

3.4. $H_2O_2$-induced $I_{CRAC}$ activation is partially mediated by IP$_3$ receptors

Next, we sought to determine the underlying mechanism of $I_{CRAC}$ activation by $H_2O_2$. First, we addressed whether $H_2O_2$ activates $I_{CRAC}$ due to store-independent and direct interaction with the pore-forming unit CRACM1.

This was investigated in HEK293 cells stably overexpressing CRACM1 with only endogenous STIM molecules present [21]. These cells produce small IP$_3$-induced $I_{CRAC}$ that is limited in size by the endogenous STIM molecules, as both CRACM and STIM proteins are needed to form CRAC channels that are regulated by store-depletion [24]. Whole-cell patch-clamp experiments were performed under conditions similar to those used in RBL-2H3 (Fig. 3). Application of 40 μM $H_2O_2$ produced a large inward current of $-26.7 \pm 10.6$ pA/pF at 300 s in STIM1-transfected CRACM1-overexpressing HEK-293 cells and no significant current response in non-transfected cells (Fig. 4A). Representative I/V relationships for these cells are shown in Fig. 4B. This indicates that $H_2O_2$ does not activate CRAC channels directly, but rather through store depletion and STIM1-dependent gating.

$H_2O_2$ can induce Ca$^{2+}$ release in a variety of cells and one proposed mechanism is through activation of IP$_3$ receptors (IP$_3$R) [36,37], leading to activation of $I_{CRAC}$ [17] (see Fig. 3A). To determine if the IP$_3$ pathway is involved in the activation of $I_{CRAC}$ by $H_2O_2$, we perfused RBL-2H3 cells with 100 μg/ml heparin, an antagonist of IP$_3$ receptors [38]. When applying 40 μM $H_2O_2$, heparin slowed the activation kinetics of $I_{CRAC}$ and reduced its amplitude as illustrated in Fig. 4C. In the presence of heparin, $I_{CRAC}$ developed with a half-maximal activation time of $313 \pm 1.8$ s compared to $281 \pm 1.2$ s in its absence, and reaching an amplitude of $-1.6 \pm 0.2$ pA/pF at 600 s compared to $-2.5 \pm 0.3$ pA/pF in controls without heparin. A representative I/V relationship of CRAC currents in the presence of heparin is shown in Fig. 4D. These results further indicate that IP$_3$R activity may be partly responsible for mediating the $H_2O_2$-activated $I_{CRAC}$, although at present we cannot distinguish between direct IP$_3$R effects and/or possible effects on IP$_3$ mobilization.

3.5. $H_2O_2$-induced activation of $I_{CRAC}$ independent of IP$_3$R

To further investigate the role of IP$_3$ receptors in $H_2O_2$-activated $I_{CRAC}$, we conducted patch-clamp experiments in a wild-type chicken DT40 B-lymphocyte cell line (DT40 WT) expressing all three IP$_3$ receptor isoforms (types I, II and III) as well as a genetically modified DT40 cell line in which all three IP$_3$ receptor isoforms are knocked out (DT40 KO) [39]. As DT40 cells develop very small $I_{CRAC}$, we optimized experimental conditions by increasing the external CaCl$_2$ concentration to 20 mM and expanding the voltage ramp to $-150$ mV, extracting current amplitudes at $-130$ mV [40]. Furthermore internal MgCl$_2$ concentration was increased to 3 mM to inhibit endogenous TRPM7 currents. In DT40 cells, TRPM2 does not contribute to the $H_2O_2$ effects, since perfusing these cells with 1 mM ADPR failed to induce any currents (Fig. S1B).

We tested the two cell lines for IP$_3$-activated $I_{CRAC}$ by perfusing cells with 20 μM IP$_3$. In WT cells, $I_{CRAC}$ developed immediately in 4 out of 4 cells reaching a plateau of $-1.2 \pm 0.2$ pA/pF at $75$ s, whereas KO cells did not show any current development (Fig. 5A). Neither WT nor KO cells developed $I_{CRAC}$ in the absence of IP$_3$ with Ca$^{2+}$ buffered to 150 nM (Fig. 5B). However, the DT40 KO cells have previously been shown to activate endogenous $I_{CRAC}$ when stores are depleted independent of IP$_3$ receptors [40]. To confirm these previous observations we applied 10 μM thapsigargin (Tg) at 60 s resulting in $I_{CRAC}$ development (Fig. 5C). Representative I/V relationships for the IP$_3$ and Tg experiments are displayed in Fig. 5D. Next, we assessed whether $H_2O_2$ could activate $I_{CRAC}$ in DT40 cells by applying 40 and 100 μM $H_2O_2$ from a wide-tipped puffer pipette at 60 s, but as this resulted in no clear $I_{CRAC}$ (data not shown), we included 40 μM $H_2O_2$ in the pipette solution. In WT cells this resulted in a slowly developing $I_{CRAC}$-like current in 4 out of 4 cells reaching an amplitude of $-1.2$ pA/pF at 300 s (Fig. 5E). In DT40 KO cells an inward current with smaller amplitude of $-0.5$ pA/pF at 300 s could be observed (Fig. 5E). This is significantly smaller than the amplitude in DT40 WT cells ($P<0.05$). The averaged cur-
The activation of TRPM2 by H$_2$O$_2$ is well established [6–8], but with the activation of I$_{CRAC}$ our results add a new aspect to the experimental use of H$_2$O$_2$. Jurkat T cells express both TRPM2 and I$_{CRAC}$ endogenously [11,33]. However, the H$_2$O$_2$-induced Ca$^{2+}$ influx observed after re-introduction of external Ca$^{2+}$ could completely be blocked by 1 mM La$^{3+}$ (Fig. 2B) and therefore is unlikely to represent Ca$^{2+}$ influx through TRPM2 channels. Two questions therefore remain, why was TRPM2 mediated Ca$^{2+}$ influx not apparent in Ca$^{2+}$ imaging experiments, and if it is a matter of concentration, at what concentration does H$_2$O$_2$ activate TRPM2 in Jurkat T cells? We set out to measure H$_2$O$_2$-activated TRPM2 currents in patch-clamp experiments. Conditions used to investigate the activation of I$_{CRAC}$ with intracellular Ca$^{2+}$ clamped to 150 nM did not cause any TRPM2 activation in addition to I$_{CRAC}$, even when applying 500 mM H$_2$O$_2$ (data not shown). We therefore left intracellular Ca$^{2+}$ unbuffered and assessed H$_2$O$_2$-induced single-channel activity that might reflect biophysical properties of TRPM2. This is possible in the whole-cell configuration because of the large single-channel conductance and characteristically long open times of TRPM2 channels [9,11,41]. Cells were kept in standard sodium solution with 1 mM CaCl$_2$ and 1 mM La$^{3+}$. Immediately after break-in, a recording with an 11 s long ramp protocol from −100 mV to 10 mV was started (Fig. 6) and 100 mM H$_2$O$_2$ was applied from the extracellular side. This produced only very few single channel openings in 3 out of 3 cells after 60 s. In two of the cells we observed activation of only 2 and 3 single channels and in another cell about 8 channels were active. Fig. 6 shows four ramp measurements from a representative recording with the activity of two single channels displaying a linear current with a reversal potential of 0 mV. We fitted the current traces of the first open channels in every cell with linear regression fits between −100 mV and 0 mV, yielding a mean single channel conductance of 65 ± 5.7 pS. This is very similar to the single channel conductances reported in Jurkat T cells when activating TRPM2 with ADPR, 67 pS, and cADPR, 69 pS [11]. These data, taken together with the measurements in intact cells (Fig. 2G), indicate that H$_2$O$_2$ is a poor activator of TRPM2 in Jurkat T cells and the contribution of H$_2$O$_2$-activated TRPM2 channels to Ca$^{2+}$ signals can therefore be regarded as negligible in this cell system.

4. Discussion

The current study provides evidence for H$_2$O$_2$ as a novel activator of the Ca$^{2+}$-selective CRAC current and thereby adds new knowledge to the involvement of H$_2$O$_2$ in regulating cellular calcium levels. Using calcium imaging and whole-cell patch-clamp...
experiments we demonstrate that endogenous \(I_{\text{CRAC}}\) can be activated by extracellular application and intracellular perfusion of micromolar concentrations of \(H_2O_2\) in Jurkat T cells, HEK293 cells, RBL-2H3 cells and DT40 B cells. This can be blocked by 1 μM LaCl3. Heparin reduced the \(H_2O_2\)-activated \(I_{\text{CRAC}}\) in RBL-2H3 cells suggesting that \(I_P\) receptors play an important role in the \(Ca^{2+}\) release that leads to activation of \(I_{\text{CRAC}}\). Furthermore electrophysiology experiments in DT40 WT and KO cells support the involvement of both \(I_P\)-dependent and -independent mechanisms. Lastly, we confirm the activation of TRPM2 ion channels by \(H_2O_2\) in Jurkat T cells, demonstrating that \(H_2O_2\) within the same cell line is capable of activating both endogenous TRPM2 and CRAC channels.

Application of \(H_2O_2\) has been reported to have different effects on ion channels, manifesting themselves as activation, potentiation or inhibition, but we are not aware of a report that would implicate activation of CRAC channels. There may be several explanations as to why other groups have not observed activation of \(I_{\text{CRAC}}\) by \(H_2O_2\) in their experiments: First, the cell type in the given experiment may not produce significant \(I_{\text{CRAC}}\). Second, endogenous \(I_{\text{CRAC}}\) is an exceedingly small current and requires optimized experimental conditions for electrophysiological detection, i.e. an enhanced \(Ca^{2+}\) gradient across the plasma membrane and buffering of cytosolic \(Ca^{2+}\) to prevent \(Ca^{2+}\)--induced inactivation. Third, due to its small amplitude, \(I_{\text{CRAC}}\) may not be detected in the presence of larger \(H_2O_2\)-activated currents.

In whole-cell patch-clamp experiments we investigated possible activation pathways of \(H_2O_2\)-induced \(I_{\text{CRAC}}\). The lack of current response in HEK293 cells stably overexpressing CRACM1 speaks against a direct activation of CRAC channels by \(H_2O_2\)). Instead, it appears that \(Ca^{2+}\) release from intracellular stores may underlie this effect, since depletion of calcium stores is the main activation mechanism of \(I_{\text{CRAC}}\) and \(H_2O_2\) has been reported to induce \(Ca^{2+}\) release via activation of \(I_P\) receptors in human platelets [36] and human endothelial cells [37]. This would also explain the observed reduction in \(H_2O_2\)-mediated \(I_{\text{CRAC}}\) amplitudes and delayed current activation kinetics when including heparin in the pipette solution. The involvement of \(I_P\) in \(H_2O_2\)-induced \(I_{\text{CRAC}}\) was further investigated in DT40 cells in which \(I_{\text{CRAC}}\)--like current development was observed in both WT and \(I_P\) KO cells when including \(H_2O_2\) in the pipette solution. Based on these findings it is tempting to propose that \(H_2O_2\) can also activate \(I_{\text{CRAC}}\) through \(I_P\)-independent pathways.

A previous report suggested that \(H_2O_2\) activates \(I_P\) receptors via oxidation of thiol groups, as a reduction in \(Ca^{2+}\) release was observed in endothelial cells when the reducing agent, diethio-ritol (DTT), was added to the bath solution [36]. The same study additionally ruled out that the \(H_2O_2\)-induced \(Ca^{2+}\) release occurred via an increased \(I_P\) production, although \(H_2O_2\) may induce \(I_P\) production through activation of tyrosine kinases [42]. The latter effect may be further amplified through \(H_2O_2\)--mediated inhibition of protein tyrosine phosphatases [43]. Our results with heparin, which partially inhibits \(H_2O_2\)-induced activation \(I_{\text{CRAC}}\) (see Fig. 4), suggest that at least part of the \(H_2O_2\) effect is mediated by \(I_P\) production, although we cannot rule out that heparin could also suppress a more direct activation of \(I_P\).

In addition to the proposed involvement of \(I_P\) in \(H_2O_2\) has also been reported to inhibit SERCA activity [36,44–46] and this could well be a contributing factor to \(Ca^{2+}\) store depletion leading to \(I_{\text{CRAC}}\). The underlying mechanisms are not clear, but it has been suggested that this also occurs via oxidation of thiol groups, as one study observed a protective effect of DTT on SERCA [36]. Another report, however, found DTT to have equivocal effects on \(H_2O_2\)-induced inhibition of SERCA [45]. In the latter, a Fenton reaction (\(Fe^{2+} + H_2O_2 \rightarrow OH^* + OH^- + Fe^{3+}\)) was used to generate hydroxyl radicals and thereby inducing oxidative stress in sarcoplasmic reticulum vesicles from rabbit skeletal muscle cells. Inhibition of SERCA could not be prevented by DTT when using \(Fe^{2+}\) in the Fenton reaction, but when replacing \(Fe^{2+}\) with \(Cu^{2+}\), inhibition was completely abolished. Inhibition of SERCA by hydroxyl radicals has also been suggested to be due to a direct attack on the ATP-binding site [46]. In conclusion, both \(I_P\) receptors and SERCA may be responsible for \(H_2O_2\)-mediated store depletion and subsequent activation of \(I_{\text{CRAC}}\).

Another possible contributor to store depletion by \(H_2O_2\) could be increased membrane permeability through other pathways. The membrane of sarcoplasmic reticulum in pig coronary artery smooth muscle cells had a higher passive \(Ca^{2+}\) permeability when exposed to \(H_2O_2\) than the plasma membrane [44], which would be in accordance with activation of \(I_{\text{CRAC}}\) by \(H_2O_2\) while still maintaining cell integrity. Additional \(Ca^{2+}\)-release channels could be involved, e.g. RyR. This release channel can be activated by \(H_2O_2\) [14] and induce \(I_{\text{CRAC}}\) in DT40 B cells [47], but RBL-2H3 cells do not express RyR [48] and it is therefore not involved in activation of \(I_{\text{CRAC}}\) in these cells. This does not exclude RyR from contributing to \(H_2O_2\)-activated \(I_{\text{CRAC}}\) in other RyR expressing cell lines, e.g. DT40 B cells. It should be noted that the \(H_2O_2\) concentration required for activation was in the millimolar range [14], which speaks against a significant contribution by RyR to the CRAC currents demonstrated in this study. It has recently been demonstrated that TRPM2 channels also function as lysosomal \(Ca^{2+}\)-release channels in β-cells [49]. Our data do not exclude that TRPM2-mediated \(Ca^{2+}\) release can contribute to the activation of \(I_{\text{CRAC}}\), but at least in Jurkat T cells, \(H_2O_2\) is a rather weak activator of TRPM2 and not likely to contribute either to \(Ca^{2+}\) release or \(Ca^{2+}\) entry via TRPM2. Another possible source for \(Ca^{2+}\) release could be mitochondria, since several studies have reported mitochondrial \(Ca^{2+}\) release by \(H_2O_2\) [36,37,50]. However, mitochondria are not considered as activators of \(I_{\text{CRAC}}\) and their role is to function as a \(Ca^{2+}\) buffer, thereby facilitating more extensive store depletion as well as reducing \(Ca^{2+}\)--dependent slow inactivation of \(I_{\text{CRAC}}\) [51]. Finally, it has been demonstrated that \(H_2O_2\) releases \(Ca^{2+}\) from a thapsigargin-insensitive non-mitochondrial \(Ca^{2+}\) store in endothelial cells [37], raising the possibility that \(H_2O_2\) could target an as yet unidentified CRAC store.

Lanthanides exert a blocking effect on several \(Ca^{2+}\)-conducting channels. Here we have used 1 μM LaCl3, a potent blocker of \(I_{\text{CRAC}}\) with an estimated \(K_d\) of 58 nM [52], to confirm the \(H_2O_2\)-induced inward current as \(I_{\text{CRAC}}\). It has been reported that exposing isolated sarcoplasmic reticulum vesicles to 15 μM LaCl3 also blocks \(I_P\) receptors [53], which could potentially lead to an indirect block of \(I_{\text{CRAC}}\). This, however, does not appear to be the case in the present study, as we did not observe any major differences in the \(Ca^{2+}\)--release response of any of the cell lines when comparing \(H_2O_2\) traces in the absence or presence of 1 μM LaCl3. In addition, \(I_P\) receptors in our cells are unlikely to have been exposed directly to significant concentrations of La3+ since La3+ does not easily cross membranes [54].

In this study we demonstrate the activation of \(I_{\text{CRAC}}\) both by external application of \(H_2O_2\) as well as by internal perfusion. Since \(H_2O_2\) appears to activate \(I_{\text{CRAC}}\) through store depletion, it likely interacts with components in intracellular stores and therefore requires \(H_2O_2\) to cross the plasma membrane. This may explain differences in activation times and \(H_2O_2\) concentrations required for activation of \(I_{\text{CRAC}}\) across different cell types, as they may differ in both membrane composition and expression of \(H_2O_2\)-transporting aquaporins. Additionally, differences in efficiency of cellular \(H_2O_2\)-eliminating mechanisms may influence the effective \(H_2O_2\) concentration obtained for the activation of \(I_{\text{CRAC}}\) in a given cell. Interestingly, we found that activation of \(I_{\text{CRAC}}\) required a much lower \(H_2O_2\) concentration than TRPM2 under optimized experimental conditions in Jurkat T cells where both mechanisms are present. This confirms that \(H_2O_2\) may in principle serve as an
activate TRPM2 currents but in Jurkat T cells it does so with much lower potency and efficacy than activating ICRAC.

This study is the first to describe the activation of ICRAC by H2O2, which adds new perspectives to the cross-talk between calcium homeostasis and ROS as well as to the use of H2O2 in experimental settings. This also means that the results of previous studies using H2O2 may have had Ca2+ contribution from ICRAC, which could potentially affect both internal Ca2+ concentration as well as inward current amplitude in patch-clamp experiments. If ICRAC activation is unwanted in experiments involving H2O2, addition of low concentrations of LaCl3 may be a useful tool until selective ICRAC inhibitors become available.

Given the broad spectrum of examples demonstrating cooperativity between H2O2 and Ca2+ it is highly probable that the findings of this study may be relevant in a number of processes, e.g. inflammation. Neutrophils produce substantial amount of H2O2 during the respiratory burst via NADPH oxidase and are highly dependent on intracellular Ca2+ as a trigger of processes such as adhesion, differentiation, chemotaxis, phagocytosis, oxidative activation and apoptosis [55]. Our findings provide a possible coupling between the H2O2 production and the cellular Ca2+ requirement. A similar scenario in which H2O2-activated ICRAC could be relevant is the respiratory burst in mast cells in which ROS production, including H2O2, is accompanied by an increase in Ca2+ via store-operated Ca2+ entry [56]. Furthermore it has been estimated that the H2O2 during inflammation reaches concentrations of 10–100 μM in the microenvironment surrounding macrophages [57]. This falls within the concentrations used in this study for activation of ICRAC. Finally, a recent study demonstrates that wound healing in zebra fish results in increased H2O2 concentration released by epithelial cells, extending as a concentration gradient 100–200 μM from the site of injury, presumably reaching concentrations of 0.5–50 μM gradually diminishing over 1–2 h [58]. The study further demonstrates that the released H2O2 functions as a chemotactic signal recruiting leukocytes to the damaged area. Although this contrasts the general concept that ROS produced during inflammation mainly originates from the respiratory burst of phagocytes, it provides an interesting scenario in which H2O2 can potentially function as a central mediator of Ca2+-dependent processes in different immune cells.

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Appendix A. Supplementary data

Supplemental data associated with this article can be found, in the online version, at doi:10.1016/j.cellcancer.2010.05.005.

References


