The whole-cell Ca\(^{2+}\) release-activated Ca\(^{2+}\) current, \(I_{\text{CRAC}}\), is regulated by the mitochondrial Ca\(^{2+}\) uniporter channel and is independent of extracellular and cytosolic Na\(^{+}\)

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**Key points**
- Ca\(^{2+}\) entry through Ca\(^{2+}\) release-activated Ca\(^{2+}\) channels activates numerous cellular responses. Under physiological conditions of weak intracellular Ca\(^{2+}\) buffering, mitochondrial Ca\(^{2+}\) uptake regulates CRAC channel activity.
- Knockdown of the mitochondrial Ca\(^{2+}\) uniporter channel prevented the development of \(I_{\text{CRAC}}\) in weak buffer but not when strong buffer was used instead.
- Removal of either extracellular or intra-pipette Na\(^{+}\) had no effect on the selectivity, kinetics, amplitude, rectification or reversal potential of whole-cell CRAC current.
- Knockdown of the mitochondrial Na\(^{+}\)–Ca\(^{2+}\)–Li\(^{+}\) exchanger did not prevent the development of \(I_{\text{CRAC}}\) in strong or weak Ca\(^{2+}\) buffer.
- Whole cell CRAC current is Ca\(^{2+}\)-selective.
- Mitochondrial Ca\(^{2+}\) channels, and not Na\(^{+}\)-dependent transport, regulate CRAC channels under physiological conditions.

**Abstract**
Ca\(^{2+}\) entry through store-operated Ca\(^{2+}\) release-activated Ca\(^{2+}\) (CRAC) channels plays a central role in activation of a range of cellular responses over broad spatial and temporal bandwidths. Mitochondria, through their ability to take up cytosolic Ca\(^{2+}\), are important regulators of CRAC channel activity under physiological conditions of weak intracellular Ca\(^{2+}\) buffering. The mitochondrial Ca\(^{2+}\) transporter(s) that regulates CRAC channels is unclear and could involve the 40 kDa mitochondrial Ca\(^{2+}\) uniporter (MCU) channel or the Na\(^{+}\)–Ca\(^{2+}\)–Li\(^{+}\) exchanger (NCLX). Here, we have investigated the involvement of these mitochondrial Ca\(^{2+}\) transporters in supporting the CRAC current \(I_{\text{CRAC}}\) under a range of conditions in RBL mast cells. Knockdown of the MCU channel impaired the activation of \(I_{\text{CRAC}}\) under physiological conditions of weak intracellular Ca\(^{2+}\) buffering. In strong Ca\(^{2+}\) buffer, knockdown of the MCU...
Introduction

CRAC channels are a major route for Ca\(^{2+}\) influx in eukaryotic cells where they regulate a variety of processes ranging from exocytosis to Ca\(^{2+}\)-dependent gene expression (Parekh, 2010). The CRAC channel is activated by depletion of the endoplasmic reticulum (ER) Ca\(^{2+}\) store (Hoth & Penner, 1992), the Ca\(^{2+}\) content of which is sensed by the ER-resident STIM proteins (Prakriya & Lewis, 2015). Physiologically, stores are depleted following stimulation of cell-surface receptors (Prakriya & Lewis, 2015). Orai1 proteins, which are the pore-forming subunits of the CRAC channel (Prakriya & Lewis, 2015). Orai1 proteins oligomerize and the aggregates migrate across the ER to reach specialized regions of junctional ER that are juxtaposed with the plasma membrane (Hogan, 2015). At these ER–plasma membrane contact sites, STIM binds to and gates open Orai1 proteins, which are the pore-forming subunits of the CRAC channel (Prakriya & Lewis, 2015). Classical biophysical studies from several groups have demonstrated that CRAC channels are very selective for Ca\(^{2+}\), with \(P_{Ca}/P_{Na} > 1000\), distinguish between different divalent cations and exhibit a minuscule unitary conductance, estimated from noise analysis to be in the low femtosiemens range (Parekh & Putney, 2005; Prakriya & Lewis, 2015).

Mechanisms that regulate CRAC channel activity impact on the time course of the Ca\(^{2+}\) signal and therefore on the extent of activation and duration of Ca\(^{2+}\)-dependent responses. Several mechanisms have been described including negative feedback by protein kinase C (Parekh & Penner, 1995) and by cytosolic Ca\(^{2+}\) itself. Ca\(^{2+}\) entry through CRAC channels activates two independent inhibitory pathways, resulting in fast and slow Ca\(^{2+}\)-dependent inactivation. Fast Ca\(^{2+}\)-dependent inactivation is a bi-exponential process developing within milliseconds of Ca\(^{2+}\) entry and is driven by Ca\(^{2+}\) microdomains near each open channel (Zweifach & Lewis, 1995a; Fierro & Parekh, 1999a).

It is a complex process with various domains of Orai1 and STIM1 implicated (Prakriya & Lewis, 2015). Slow Ca\(^{2+}\)-dependent inactivation develops over tens of seconds, requires a rise in bulk cytosolic Ca\(^{2+}\) and involves the ER-resident protein SARAF (Zweifach & Lewis, 1995b; Parekh, 1998; Palty et al. 2012).

Mitochondria are also important regulators of CRAC channel activity through buffering of cytosolic Ca\(^{2+}\) (Rizzuto & Pozzan, 2006). Ca\(^{2+}\) entry into mitochondria is driven by the large negative potential across the inner mitochondrial membrane and occurs through the mitochondrial Ca\(^{2+}\) uniporter (MCU), a Ca\(^{2+}\)-selective inwardly rectifying channel spanning the inner mitochondrial membrane (Baughman et al. 2011; De Stefani et al. 2011). Other Ca\(^{2+}\) uptake pathways have also been reported, including one involving uncoupler proteins 2 and 3 (Trenker et al. 2007). In RBL mast cells, we have shown that mitochondria buffer InsP\(_3\)-dependent Ca\(^{2+}\) release and this leads to more extensive store depletion (Gilbert & Parekh, 2000; Samanta et al. 2014). Mitochondria also buffer Ca\(^{2+}\) entry, reducing the rate and extent of Ca\(^{2+}\)-dependent slow inactivation of the channels (Gilbert & Parekh, 2000; Hoth et al. 2000; Glitsch et al. 2002b). However, the molecular identity of the mitochondrial Ca\(^{2+}\) channel necessary for supporting \(I_{CRAC}\) is unknown. Mitochondria can extrude Ca\(^{2+}\) from the matrix through the activities of two exchangers, the Ca\(^{2+}\)-H\(^{+}\) exchanger LetM1 (Jiang et al. 2009) and the Na\(^{+}\)-Ca\(^{2+}\)-Li\(^{+}\) exchanger (NCLX) (Palty et al. 2010). Recently, it has been proposed that mitochondrial Ca\(^{2+}\) extrusion mediated by NCLX is essential for the activation of \(I_{CRAC}\) following store depletion (Ben-Kassus Nissim et al. 2017). This mechanism was observed in high cytosolic BAPTA, suggesting it was independent of mitochondrial Ca\(^{2+}\) buffering. It was also proposed that store depletion activated plasma membrane Na\(^{+}\) buffer. It was also proposed that store depletion activated plasma membrane Na\(^{+}\) channel in parallel with \(I_{CRAC}\) with the former driving the rise in cytosolic Na\(^{+}\) needed to stimulate mitochondrial NCLX and thus enabling \(I_{CRAC}\) to develop (Ben-Kassus Nissim et al. 2017). Consequently, the whole-cell CRAC

channel did not inhibit \(I_{CRAC}\) development demonstrating that mitochondria regulate CRAC channels under physiological conditions by buffering of cytosolic Ca\(^{2+}\) via the MCU channel. Surprisingly, manipulations that altered extracellular Na\(^{+}\), cytosolic Na\(^{+}\) or both failed to inhibit the development of \(I_{CRAC}\) in either strong or weak intracellular Ca\(^{2+}\) buffer. Knockdown of NCLX also did not affect \(I_{CRAC}\). Prolonged removal of external Na\(^{+}\) also had no significant effect on store-operated Ca\(^{2+}\) entry, on cytosolic Ca\(^{2+}\) oscillations generated by receptor stimulation or on CRAC channel-driven gene expression. In the RBL mast cell, Ca\(^{2+}\) flux through the MCU but not NCLX is indispensable for activation of \(I_{CRAC}\).
current would involve a Na\(^+\) current, which questions earlier conclusions on the selectivity of the CRAC current. In this study, we address the role of the MCU channel and both extracellular and cytosolic Na\(^+\) in the regulation of \(I_{\text{CRAC}}\), Ca\(^{2+}\) signalling and CRAC channel-driven gene expression. We find a central role for the MCU but not for extracellular or intracellular Na\(^+\) in supporting CRAC channel activity under physiological conditions.

**Methods**

**Cell culture**

Rat basophilic leukemia (RBL-2H3) cells were purchased from ATCC (Manassas, VA, USA). Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum and 1% penicillin/streptomycin, as previously described (Samanta et al. 2014).

**Patch clamp recordings**

Patch clamp experiments were conducted in the tight seal whole-cell configuration at room temperature (20–24°C) as previously described (Glitsch et al. 2002b). Pipettes were pulled from borosilicate glass and were Sylgard-coated and fire polished. Pipettes had resistances of 3–6 MΩ when filled with the various internal solutions used for \(I_{\text{CRAC}}\) measurements (Table 1). The CRAC current was measured by applying voltage ramps (−100 to +100 mV in 50 ms) at 0.5 Hz from a holding potential of 0 mV. Current through Kv1.5 channels was measured using voltage ramps spanning −100 to +100 mV in 250 ms, applied at 0.2 Hz to allow for recovery from C-type inactivation between ramps. Holding potential for recording of Kv1.5 was −80 mV. Currents were filtered using an eight-pole Bessel filter at 2.5 kHz and digitized at 100 μs. Capacitative currents were compensated before each ramp or step by using the automatic compensation of the EPC 9-2 amplifier (HEKA, Lambrecht/Pfalz, Germany). Leak currents were subtracted by averaging three to five ramp currents obtained just before \(I_{\text{CRAC}}\) had started to develop (passive store depletion) or by subtracting the first one or two ramp currents (active store depletion with Ins\(_3\)). Recordings were made under paired conditions, in that test conditions and controls were carried out on alternate coverslips on the same days.

The solutions used are listed in Table 1.

**23Na nuclear magnetic resonance measurements**

All sodium nuclear magnetic resonance (NMR) data were acquired at 132 MHz at room temperature using a Bruker Avance III NMR spectrometer (Billerica, MA, USA) equipped with a TBO probe. All experiments used a

| Table 1. Solutions used |
|--------------------------|
| **Solution**               | **Composition (mM)**                                      |
| Internal solutions        |                                                        |
| Na\(^+\) containing, active store depletion | 145 Cs-glutamate, 8 NaCl, 1 MgCl\(_2\), 2.8 KCl, 2 pyruvic acid, 2 K-malate, 1 KH\(_2\)PO\(_4\), 2 Mg-ATP, pH 7.2 (CsOH) |
| Na\(^+\) free, active store depletion | 145 Cs-glutamate, 8 CsCl, 1 MgCl\(_2\), 2 pyruvic acid, 2 K-malate, 1 KH\(_2\)PO\(_4\), 2 Mg-ATP, pH 7.2 (CsOH) |
| Na\(^+\) containing, passive store depletion | 145 Cs-glutamate, 8 NaCl, 1 MgCl\(_2\), 10 HEPES, 10 EGTA, pH 7.2 (CsOH) |
| Na\(^+\) free, passive store depletion | 145 Cs-glutamate, 8 CsCl, 1 MgCl\(_2\), 10 HEPES, 10 EGTA, pH 7.2 (CsOH) |
| Na\(^+\) containing, mitochondrial cocktail | 145 Cs-glutamate, 8 NaCl, 1 MgCl\(_2\), 2 pyruvic acid, 2 K-malate, 1 KH\(_2\)PO\(_4\), 2 Mg-ATP, pH 7.2 (CsOH) |
| Na\(^+\) free, mitochondrial cocktail | 145 Cs-glutamate, 8 CsCl, 1 MgCl\(_2\), 2 pyruvic acid, 2 K-malate, 1 KH\(_2\)PO\(_4\), 2 Mg-ATP, pH 7.2 (CsOH) |
| Kv1.5, K\(^+\) based | 145 K-glutamate, 8 NaCl, 1 MgCl\(_2\), 10 HEPES, 0.35 EGTA, 2 Mg-ATP, pH 7.2 (KOH) |
| Kv1.5, Cs\(^+\) based | 145 Cs-glutamate, 8 NaCl, 1 MgCl\(_2\), 10 HEPES, 0.35 EGTA, 2 Mg-ATP, pH 7.2 (CsOH) |
| Extracellular solutions for patch clamp recordings |                                                        |
| Na\(^+\) containing | 155 NaCl, 10 CaCl\(_2\), 10 CsCl, 2.8 KCl, 2 MgCl\(_2\), 10 HEPES, 10 glucose, pH 7.4 (NaOH) |
| Na\(^+\) free | 155 Tris-Cl, 10 CaCl\(_2\), 10 CsCl, 2.8 KCl, 2 MgCl\(_2\), 10 HEPES, 10 glucose, pH 7.4 (NaOH) |
| Ca\(^{2+}\) free | Ca\(^{2+}\)-free solution was made by omitting CaCl\(_2\) from the above solutions |
| Kv1.5 | 155 NaCl, 2 CsCl, 10 CsCl, 2.8 KCl, 2 MgCl\(_2\), 10 HEPES, 10 glucose, pH 7.4 (NaOH) |
| Extracellular solutions for fluorescence measurements |                                                        |
| Na\(^+\) containing | 155 NaCl, 2 CaCl\(_2\), 2.8 KCl, 2 MgCl\(_2\), 10 HEPES, 10 glucose, pH 7.4 (NaOH) |
| Na\(^+\) free | 155 Tris-Cl, 2 CaCl\(_2\), 2.8 KCl, 2 MgCl\(_2\), 10 HEPES, 10 glucose, pH 7.4 (HCl) |
| Ca\(^{2+}\) free | Ca\(^{2+}\)-free solution was made by omitting CaCl\(_2\) from the above solutions followed by addition of 0.1 mM EGTA. pH was returned to 7.4 with either NaOH or HCl. |
standard 90-degree pulse-acquire protocol with constant parameters, including number of scans, receiver gain and pulse length. All samples for NMR were prepared to a final volume of 0.6 mL in 5 mm NMR tubes. Calibration curve samples were prepared in 100% D$_2$O, and samples for analysis were prepared with 10% D$_2$O.

A calibration curve for free sodium concentration was generated using five different NaCl samples with sodium concentrations in the range 0.02–340 mM. Peak integrals were plotted against concentration, and the calibration curve showed excellent linearity ($R^2 = 0.9998$). To determine the free sodium concentration in a sample, the sodium NMR peak was integrated and fitted to the curve.

### Cytosolic Ca$^{2+}$ measurements

Cytosolic Ca$^{2+}$ measurements were carried out at room temperature using the IMAGO charge-coupled device camera-based system from TILL Photonics (now FEI GmbH, Munich, Germany), as described previously (Samanta et al. 2014). Cells were loaded with Fura-2/AM (1 μM; Molecular Probes/Thermo Fisher Scientific, Waltham, MA, USA) for 40 min in the dark at room temperature and then washed three times in standard external solution (Table 1). Cells were left for 15 min to allow further de-esterification. Cells were alternately excited at 356 nm and 380 nm (20 ms exposures) at 0.5 Hz. Analysis was performed offline using Igor Pro for Windows (WaveMetrics, Lake Oswego, OR, USA). Ca$^{2+}$ signals are plotted as $R$, denoting the 356/380 ratio.

### Cytosolic Na$^+$ measurements

Cells were loaded with CoroNa Green-AM (5 μM; Invitrogen, Carlsbad, CA, USA) for 45 min at room temperature in the dark in either standard Na$^+$-containing external solution or Na$^+$-free external solution, as described in the text and then washed three times in the appropriate Na$^+$-containing or Na$^+$-free solution. Cells were left for 15 min to allow further de-esterification. Cells were excited at 490 nm (20 ms exposures) and images were acquired every 3 s. Images were analysed offline using Igor Pro. The Na$^+$ signals were normalized to the averaged baseline signal ($F/F_0$), obtained at the beginning of the measurements. In some experiments, gramicidin A (Santa Cruz Biotechnology, Dallas, TX, USA; cat. no.: SC-203061) was added to raise cytosolic Na$^+$. In some cells, CoroNa green signals bleached rapidly. Therefore, we measured the background rate of bleaching prior to stimulation and used only those cells which showed a gradual decay in fluorescence prior to stimulation. Two to three cells were used per field of view and therefore $n = 9$ (see text) indicates data from three to four separate experiments.

### Cell transfection and small interfering RNA

RBL cells were transfected using the AMAXA system, using nucleofector cell line kit V solution (from Lonza, Slough, UK, cat. no. VCA-1003) and program T-30 as described (Samanta et al. 2014).

Rat MCU SiRNA was from Origene (Rockville, MD, USA, cat. no.: SR508660) and had the following sequence:

- SR508660A – rCrCrUrArGrArGrArArUrCrArArUrCraAA GrUrArGrArGrArCTG
- SR508660B – rGrGrCrArGrArArUrGrGrArUrCrUr UrArGrArGrArCTG
- SR508660C – rGrCrCrGrArGrArCrGrArCrArCrAr UrCrUrUrUrUAT

Rat NCLX small interfering RNA (siRNA) was from Invitrogen (cat. no. 10620310) and had the following sequence: AACGGCCACUCACUGUCU.

Kv1.5 was a kind gift from Prof. Stefan Heinemann (Friedrich-Schiller-Universität Jena, Jena).

### c-fos RT-PCR

RBL cells were initially placed in either Na$^+$-containing or Na$^+$-free extracellular solution for 1 h (see text). Cells that had been placed in Na$^+$-free solution were then stimulated with a submaximal dose of leukotriene C$_4$ (LTC$_4$) (160 nM) or 2 μM thapsigargin for 10 min in Na$^+$-free solution and then washed with Na$^+$-free and Ca$^{2+}$-free solution (containing 0.1 mM EGTA) without stimulus for a further 40 min. For cells placed in Na$^+$-containing external solution, stimulation occurred in Na$^+$-containing solution for 10 min and then cells were washed with Na$^+$-containing and Ca$^{2+}$-free solution (with 0.1 mM EGTA) for 40 min without stimulus. Thereafter, for both groups (exposed to Na$^+$-containing or Na$^+$-free solution), total RNA was extracted by using an RNasy Mini Kit (Qiagen, Hilden, Germany) (Ng et al. 2009). RNA was quantified spectrophotometrically by absorbance at 260 nm. Total RNA (1 μg) was reverse-transcribed using the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA), according to the manufacturer’s instructions. Following cDNA synthesis, PCR amplification was performed using BIOX-ACT™ ShortDNAPolymerase (Biolone) with primers specific for the detection of c-fos and β-actin from Invitrogen:

- c-Fos; forward: 5′ AGCCGACTCTCTTCCAGCAT 3′
- c-Fos; reverse: 5′ CAGATAGCTGCTCTACTTTGC 3′
- β-actin; forward: 5′ TTGTAAACCAACTGGGACGAT ATG 3′
- β-actin; reverse: 5′ GATCTTGATCTTCATGGTGC AGG 3′

The PCR products were electrophoresed through an agarose gel and visualized by ethidium bromide staining.
Gene expression assay

Twenty-four hours after transfection with enhanced green fluorescent protein (EGFP)-based reporter plasmid containing an NFAT promoter (gift from Dr Yuri Usachev, Iowa University), cells were placed in either Na\(^+\)-containing or Na\(^+\)-free extracellular solution for 1 h in an incubator and then stimulated with LTC\(_4\) or thapsigargin (100 nM) for 10 min in appropriate Na\(^+\)-containing or Na\(^+\)-free extracellular solution. Control groups had the same treatment but without stimulus. Cells were then washed to remove agonist and then maintained in either Na\(^+\)-containing or Na\(^+\)-free extracellular solution for a further 30 min (a time within which cells had recovered fully from stimulation with LTC\(_4\), as evidenced by a return to a stable resting cytosolic Ca\(^{2+}\) level) before washing with DMEM. Cells were left in the incubator for ~24 h prior to detection of EGFP, as described (Kar et al. 2012b).

Statistics

Statistical significance was calculated using the Instat 2.03 programme (for Macintosh; GraphPad Software, La Jolla, CA, USA). Comparison of two means was evaluated by Student’s unpaired, two-tailed t test. For comparison of more than two means, a two-tailed ANOVA was used and the Student–Newman–Keuls or Bonferroni test was used for post hoc analyses. Data are presented as means ± SEM. The number of cells analysed is given in the figure legend and represents combined data from at least two independent cell preparations. Level of significance is shown as *P < 0.01 and ** P < 0.001.

Results

I\(_{CRAC}\) activated following store depletion with InsP\(_3\) is unaffected by removal of external Na\(^+\)

To see whether extracellular Na\(^+\) was required for, or contributed to, I\(_{CRAC}\), we placed cells in Na\(^+\)-free external solution for at least 15 min prior to the onset of whole-cell patch clamp recording. Stores were depleted by inclusion of InsP\(_3\) together with 10 mM EGTA in a Na\(^+\)-containing patch pipette solution. In control cells maintained in Na\(^+\)-containing external solution, store depletion resulted in the development of I\(_{CRAC}\) (Fig. 1A, black trace). The current–voltage (I–V) relationship exhibited the hallmarks of I\(_{CRAC}\) including non-voltage-dependent gating, marked inward rectification and a very positive reversal potential (Fig. 1B). The current activated mon-exponentially with a time constant of ~25 s (Fig. 1C) to reach an amplitude of ~3.5 pA/pF (Fig. 1D). The extent of rectification by plotting the size of the current at ~80, ~40 and 0 mV (Fig. 1E). The reversal potential of the current was ~+90 mV (Fig. 1F). In the absence of external Na\(^+\), I\(_{CRAC}\) developed in a manner that was qualitatively very similar to that seen in the presence of external Na\(^+\) (Fig. 1A, red trace). The I–V relationship (Fig. 1B), the mono-exponential activation time constant (Fig. 1C), the amplitude of the current (Fig. 1D), the extent of rectification (Fig. 1E) and the reversal potential (Fig. 1F) were all indistinguishable from responses obtained in the presence of external Na\(^+\).

Removal of intracellular Na\(^+\) does not affect I\(_{CRAC}\)

Although the preceding experiments show that removal of external Na\(^+\) has no discernible impact on I\(_{CRAC}\), we hypothesized that the Na\(^+\) concentration in our pipette solution was sufficient to maintain cytosolic Na\(^+\) at the level needed to support the current. To test this, we repeated these experiments but now in the complete absence of Na\(^+\). We simultaneously used Na\(^+\)-free pipette and extracellular solutions. Under these conditions, I\(_{CRAC}\) developed with virtually identical features of time course, I–V relationship, amplitude, extent of rectification and reversal potential compared with cells dialysed with Na\(^+\)-containing external and pipette solutions (Fig. 1A–F). None of the parameters measured were significantly affected by the absence of either extracellular Na\(^+\) or both extracellular and intracellular Na\(^+\). I\(_{CRAC}\) therefore activates robustly even when Na\(^+\) is absent simultaneously from extracellular and pipette solutions.

Na\(^+\) concentrations in the solutions

To confirm our Na\(^+\)-free solutions lacked Na\(^+\), we measured the Na\(^+\) concentrations using \(^{23}\)Na NMR. A strong sodium signal was detected in Na\(^+\)-containing external solution (Fig. 2A) but no signal was seen in Na\(^+\)-free external solution (Fig. 2B). A Na\(^+\) signal was also seen in our Na\(^+\)-containing pipette solution (Fig. 2C) and this was almost abolished in the Na\(^+\)-free pipette solution (Fig. 2D). We constructed a calibration curve (Fig. 2E), and found the peak area of the \(^{23}\)Na NMR signal was linearly proportional to the ambient Na\(^+\) concentration over the range 1–350 mM. Our Na\(^+\)-containing external solution had a Na\(^+\) concentration of ~169 mM (Fig. 2F). Importantly, no Na\(^+\) was detected in our Na\(^+\)-free external solution indicating a Na\(^+\) concentration <1 mM (Fig. 2F). Our internal solution contained 8.1 mM Na\(^+\) (expected Na\(^+\) was 8 mM) and our Na\(^+\)-free pipette solution contained < 1 mM (Fig. 2F). Therefore our Na\(^+\)-free solutions indeed were devoid of Na\(^+\).

Time course of washout of a monovalent cation

Freely diffusible ions or molecules that are present in the cytosol but omitted from the pipette solution will
dialyse or wash out from the cytosol at a rate determined by the series resistance, cell size and molecular mass of the substance (Pusch and Neher, 1988). When Na\(^+\) is absent from the pipette solution, as was the case in the preceding experiments, the time constant for washout is expected to be fast, being \(\sim 30\) s with our typical series resistance of 8 M\(\Omega\) and cell size of 10 pF. RBL cells, like many non-excitable cells, do not express voltage-gated Na\(^+\) channels and therefore it is not possible to measure the time course of Na\(^+\) dialysis through a shift in reversal potential of such channels. RBL cells do express a GTP-activated Na\(^+\) channel but the current develops slowly over several minutes (Parekh, 1996), precluding a measure of the rate of Na\(^+\) washout. To estimate the kinetics of dialysis of a monovalent cation between cytosol and pipette solution, we therefore measured the rate of loss of K\(^+\) ions as gauged through the rate of rundown of voltage-gated Kv1.5 currents following whole-cell dialysis with a K\(^+\)-free pipette solution (potassium glutamate had been replaced by caesium glutamate) compared with a K\(^+\)-rich solution. Expression of Kv1.5 channels resulted in large outward K\(^+\) currents, measured using a voltage ramp protocol (Fig. 3A). These currents were relatively stable because repetitive voltage ramps evoked similarly sized currents. Currents to the first four ramps after break-in with a K\(^+\)-rich pipette solution are shown in Fig. 3A along with that to the 30th ramp. The time course for this whole-cell current (measured at +80 mV) is shown in Fig. 3C. The K\(^+\) current was stable, declining \(< 10\%\) after 100 s of whole-cell dialysis. Aggregate data comparing the extent of decline of the current with the time constant taken to reach the steady state level is shown in Fig. 3D. The currents ran down considerably more quickly when K\(^+\) was replaced by Cs\(^+\) (Fig. 3B–D). Rundown was rapid

**Figure 1. Impact of changing extracellular and intra-pipette Na\(^+\) on \(I_{CRAC}\) evoked by Ins\(_P\)\(_3\)**

A, time course of \(I_{CRAC}\) compared in different cells exposed to the different Na\(^+\) solutions indicated. +Na\(^+\)_o; +Na\(^+\)_i (black trace) denotes Na\(^+\)-containing external and pipette solutions. 0Na\(^+\)_o; +Na\(^+\)_i (red trace) denotes Na\(^+\)-free external solution and Na\(^+\)-containing pipette solution. 0Na\(^+\)_o; 0Na\(^+\)_i (green trace) denotes Na\(^+\)-free external and pipette solutions. The cell in 0Na\(^+\)_o; +Na\(^+\)_i was in Na\(^+\)-free solution for 27 min prior to break-in. The cell in 0Na\(^+\)_o; 0Na\(^+\)_i was in Na\(^+\)-free solution for 19 min before break-in. B, \(I-V\) relationships compared for the conditions indicated, taken once the whole-cell currents in panel A had peaked. C, mono-exponential time constant of activation of \(I_{CRAC}\) compared for the conditions shown. D, peak amplitude of \(I_{CRAC}\) compared for conditions indicated. E, the extent of inward rectification over the voltage range −80 mV to 0 mV depicted for the various conditions. F, bar chart comparing the reversal potential of \(I_{CRAC}\) for the different conditions. Averaged data for +Na\(^+\)_o; +Na\(^+\)_i was from 12 cells, 0Na\(^+\)_o; +Na\(^+\)_i was from 9 cells and 0Na\(^+\)_o; 0Na\(^+\)_i was from 10 cells. There were no statistically significant differences between any of the groups.

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but reached a steady state where small Cs\(^+\) currents could be measured, reflecting the low \(P_{Cs}/P_K\) of the channels (Fig. 3B and D; Lin et al. 2001). Interaction between K\(^+\) and Cs\(^+\) shortly after break-in likely accounts for the biphasic current that was seen only in the initial ramp currents (Fig. 3B). Therefore monovalent group 1 cations like K\(^+\) and Cs\(^+\) exchange quickly between cytosol and pipette and, when omitted from the pipette solution, the smaller Na\(^+\) should wash out rapidly within a few tens of seconds.

**Figure 2. Measurement of Na\(^+\) in solutions using \(^{23}\)Na-NMR**

A, spectrum for \(^{23}\)Na in standard external solution. B, \(^{23}\)Na spectrum in Na\(^+\)-free external solution. C, \(^{23}\)Na spectrum in standard Na\(^+\)-containing pipette solution. D, \(^{23}\)Na spectrum in Na\(^+\)-free pipette solution. E, calibration curve measuring peak area for different Na\(^+\) concentrations. Data from panels A and C are included. F, summary of Na\(^+\) concentrations in our solutions.

|                | Expected | Measured (mM) |
|----------------|----------|---------------|
| **External Solution** |          |               |
| Na\(^+\)-containing | ~162     | 169±10        |
| Na\(^+\)-free       | ~0       | <<1           |
| **Internal Solution** |          |               |
| Na\(^+\)-containing  | 8        | 8.1±0.5       |
| Na\(^+\)-free       | ~0       | <1            |

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Robust $I_{\text{CRAC}}$ activation by passive store depletion in the absence of Na$^+$

The expected $\tau_{\text{washout}}$ of cytosolic Na$^+$ is $\sim30$ s, when RBL cells are dialysed with our Na$^+$-free ($<1$ mM) pipette solution. Because $I_{\text{CRAC}}$ activates following dialysis with InsP$_3$ with a delay $<2$ s and with a time constant of activation of $\sim25$ s (Fig. 1C), the current develops following dialysis with a Na$^+$-free pipette solution as intracellular free Na$^+$ is declining quickly but is nevertheless still present. In order to activate $I_{\text{CRAC}}$ after cytosolic Na$^+$ had washed out of the cytosol, we depleted stores passively by dialysing cells with a pipette solution containing 10 mM EGTA but without InsP$_3$. Passive store depletion with EGTA activates $I_{\text{CRAC}}$ slowly (Fierro & Parekh, 1999b); following a delay of $\sim80$ s, the current develops gradually, taking a further $\sim200$ s before reaching its peak value. Therefore with passive store depletion, cytosolic Na$^+$ would have washed out of the cell following dialysis with a Na$^+$-free pipette solution well before $I_{\text{CRAC}}$ activates.

In control cells exposed to Na$^+$-containing extracellular solution and dialysed with a pipette solution containing Na$^+$, $I_{\text{CRAC}}$ activated slowly as expected for passive store depletion (Fig. 4A, black trace). The $I$–$V$ relationship exhibited the characteristics of $I_{\text{CRAC}}$ (Fig. 4B). The current reached an amplitude of $\sim3$ pA/pF (Fig. 4C), similar to that evoked by InsP$_3$ (Fig. 1D). $I_{\text{CRAC}}$ developed gradually, exhibiting a delay of $\sim75$ s (Fig. 4D) and the subsequent time taken for the current to peak was $\sim200$ s (Fig. 4E). The extent of rectification (Fig. 4F) and reversal potential (Fig. 4G) were similar to those seen when InsP$_3$ was used to deplete the stores instead (Fig. 1E and F).

We systematically removed extracellular Na$^+$ and pipette Na$^+$ to see whether these manoeuvres affected any of the properties of $I_{\text{CRAC}}$. Removal of pipette Na$^+$, leaving extracellular Na$^+$ present, or removal of extracellular Na$^+$ leaving pipette Na$^+$ intact, had no effect on any of the properties of the current activated by passive store depletion (Fig. 4A–G). We also activated $I_{\text{CRAC}}$ through passive store depletion in the simultaneous absence of intra- and extracellular Na$^+$. In cells pre-exposed to Na$^+$-free external solution for 15–30 min and then dialysed with a Na$^+$-free pipette solution, $I_{\text{CRAC}}$ was indistinguishable from that seen in the presence of extracellular and intra-pipette Na$^+$ (Fig. 4A–G). None of the kinetics of activation, the $I$–$V$ relationship, the amplitude, the extent of rectification of the current or the reversal potential were affected by the simultaneous removal of Na$^+$ from both extracellular and pipette solutions.

Ca$^{2+}$-dependent fast inactivation of CRAC channels

Another hallmark of CRAC channels is that they exhibit Ca$^{2+}$-dependent fast inactivation whereby Ca$^{2+}$ ions that have permeated a channel feed back to reduce further channel activity. Fast inactivation develops along a biexponential time course during hyperpolarizing pulses below $-40$ mV. In RBL cells, we have previously characterized fast inactivation in detail (Fierro & Parekh, 1999a) and found it exhibits identical features to those first described in T cells (Zwiebatch and Lewis, 1995a). If a Na$^+$ current developed in parallel with $I_{\text{CRAC}}$, a simple prediction would be that the rate and/or extent
of fast inactivation of $I_{\text{CRAC}}$ should be altered by removal of external $\text{Na}^+$ as it is unlikely the $\text{Na}^+$-permeable channels would show identical rates and extents of fast inactivation to those of CRAC channels. To test this, we compared fast inactivation between cells exposed to $\text{Na}^+$-containing extracellular solution with those maintained in $\text{Na}^+$-free solution for several minutes prior to the onset of experiments. $\text{Na}^+$-free pipette solution was used in both cases. Fast inactivation was induced by application of voltage steps ($-40$ mV to $-120$ mV in $20$ mV increments, $250$ ms duration). In control cells maintained in $\text{Na}^+$-containing solution, hyperpolarizing pulses below $-40$ mV elicited fast inactivation which increased with stronger hyperpolarization (Fig. 5A). The extent of fast inactivation increased monotonically with hyperpolarizing pulse potential (Fig. 5B) and could be fitted with a Boltzmann-type equation (where $R$, $T$, $Z$ and $F$ have their usual meanings)

$$\text{CDI} = \frac{\text{CDI}_{\text{max}}}{1 - \exp \left( \frac{Z}{RT} \left( \frac{V}{V_{1/2}} \right) \right)}$$

yielding $V_{1/2}$ of $-68$ mV (Fig. 5B). Inactivation followed a biexponential process, with time constants of $\sim 10$ and $\sim 100$ ms at $-120$ mV for the fast and slow components, respectively (Fig. 5C and D).

In $\text{Na}^+$-free external solution, fast inactivation showed a superimposable dependence on hyperpolarizing pulse potential (Fig. 5B), with a $V_{1/2}$ of $-66$ mV. The biexponential kinetics of inactivation in $\text{Na}^+$-free solution were also similar to those seen in $\text{Na}^+$-containing solution (Fig. 5C and D).

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**Figure 4. Effects of varying extracellular and intra-pipette $\text{Na}^+$ on $I_{\text{CRAC}}$ evoked by passive store depletion by dialysis with high EGTA-containing pipette solution**

**A**, time course of $I_{\text{CRAC}}$ compared in the presence of different external and intra-pipette $\text{Na}^+$ concentrations. **B**, steady-state $I$–$V$ curves taken from experiments in panel A. **C**, peak amplitude of $I_{\text{CRAC}}$ compared for the conditions shown. **D**, delay before $I_{\text{CRAC}}$ activates compared for the conditions indicated. **E**, time to peak (measured from the delay) shown for the various experimental conditions. **F**, inward rectification, over the voltage range $-80$ to $0$ mV is compared. **G**, bar chart showing reversal potential of the whole-cell current for the different conditions. Averaged data for $+\text{Na}_0^+$; $+\text{Na}_i^+$ was from 12 cells, $0\text{Na}_0^+$; $-\text{Na}_i^+$ was from 10 cells, $+\text{Na}_0^+$; $0\text{Na}_i^+$ was from 8 cells and $0\text{Na}_0^+$; $0\text{Na}_i^+$ was from 11 cells. There were no statistically significant differences between the groups.

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**$I_{\text{CRAC}}$ activates after extensive dialysis of the cytosol with Na\textsuperscript{+}-free solutions**

We designed experiments to activate $I_{\text{CRAC}}$ after extensive dialysis of the cytosol with Na\textsuperscript{+}-free pipette solution under physiological conditions of weak intracellular Ca\textsuperscript{2+} buffering. First, cells were placed in Na\textsuperscript{+}- and Ca\textsuperscript{2+}-free external solution supplemented with the sarco/endo-plasmic reticulum Ca\textsuperscript{2+}-ATPase (SERCA) pump blocker thapsigargin (2 μM). By blocking SERCA pumps, thapsigargin prevents store refilling and, in the continuous presence of Ca\textsuperscript{2+} leak out of the ER, the stores deplete and CRAC channels open. But in the absence of external Ca\textsuperscript{2+}, no current flows. Cells were dialysed with a Na\textsuperscript{+}-free pipette solution containing weak Ca\textsuperscript{2+} buffer supplemented with a mitochondrial cocktail solution that maintains the organelle in an energized state and therefore sustains mitochondrial Ca\textsuperscript{2+} buffering (Gilabert & Parekh, 2000). We selected cells with a low series resistance (typically <8MΩ) and small size (input capacitance typically <10 pF) in order to achieve rapid dialysis of Na\textsuperscript{+} from the cytosol. Following break-in, no current developed. We dialysed a cell with a Na\textsuperscript{+}-free pipette solution for ~220 s (~8 times $\tau_{\text{washout}}$ of cytosolic Na\textsuperscript{+}) before readmitting external Ca\textsuperscript{2+}; a robust CRAC current was still produced (Fig. 6A; blue trace). The $I$–$V$ relationship is shown in Fig. 6B. Control cells dialysed with Na\textsuperscript{+}-containing external and pipette solutions generated $I_{\text{CRAC}}$ (Fig. 6A) with a very similar $I$–$V$ relationship (Fig. 6B) and a similar peak amplitude (Fig. 6C) to that seen when the current was evoked in Na\textsuperscript{+}-free solutions. Therefore, despite extensive dialysis with Na\textsuperscript{+}-free pipette solution in the absence of external Na\textsuperscript{+}, $I_{\text{CRAC}}$ development is not impaired in weak intracellular Ca\textsuperscript{2+} buffer.

**$I_{\text{CRAC}}$ activation under physiological conditions of weak intracellular Ca\textsuperscript{2+} buffering is independent of intra- and extracellular Na\textsuperscript{+}**

Under physiological conditions of weak intracellular Ca\textsuperscript{2+} buffering, Ins\textsubscript{3} activates $I_{\text{CRAC}}$ provided mitochondrial Ca\textsuperscript{2+} uptake is maintained by inclusion of the mitochondrial cocktail (Gilabert & Parekh, 2000). To test whether intra- or extracellular Na\textsuperscript{+} was required to support $I_{\text{CRAC}}$ under physiological conditions of weak buffering and with Ins\textsubscript{3} as the stimulus to deplete stores, we examined the ability of Ins\textsubscript{3} to activate the current in the absence of both extra- and intracellular Na\textsuperscript{+}. Dialysis with Ins\textsubscript{3} activated $I_{\text{CRAC}}$ in Na\textsuperscript{+}-containing external and pipette solutions (Fig. 7A). The $I$–$V$ relationship was typical of $I_{\text{CRAC}}$ (Fig. 7B). $I_{\text{CRAC}}$ activated with a mono-exponential time constant (Fig. 7C) to reach a peak amplitude of ~1.5 pA/pF (Fig. 7D). This is significantly less than that seen in strong buffer (Fig. 1D) and reflects incomplete store depletion due to active SERCA pumps (Glitsch et al. 2002b). Robust $I_{\text{CRAC}}$ also developed in cells exposed to Na\textsuperscript{+}-free extracellular solution and dialysed with a Na\textsuperscript{+}-free pipette solution (Fig. 7A), and the $I$–$V$ relationship (Fig. 7B), the activation kinetics (Fig. 7C) and the amplitude of the current (Fig. 7D) were all similar to those seen in Na\textsuperscript{+}-containing solutions. Dialysis with a weak buffer-based internal solution containing cocktail but no Ins\textsubscript{3} failed to activate any inward current (Fig. 7A and D), confirming the development of the current was due to store depletion by Ins\textsubscript{3}.

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**Figure 5. Effect of removing Na\textsuperscript{+} on Ca\textsuperscript{2+}-dependent fast inactivation of CRAC channels**

A, fast inactivation compared following hyperpolarizing pulses to ~120 and ~80 mV. B, aggregate data comparing the percentage of fast inactivation with hyperpolarizing pulse potential compared for the two conditions shown. Each point is the mean of 5 cells. Extent of fast inactivation was assessed as (1 – steady-state current at end of pulse/peak current) $\times$ 100%. C, graph comparing the fast time constant of fast inactivation between Na\textsuperscript{+}-containing and Na\textsuperscript{+}-free solutions. D, graph comparing the slow time constant of fast inactivation.

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Store-operated Ca\(^{2+}\) entry in intact cells in the absence of external Na\(^+\)

In fura 2-loaded cells, stimulation with thapsigargin in Ca\(^{2+}\)-free external solution leads to a transient rise in cytosolic Ca\(^{2+}\) as Ca\(^{2+}\) is released from the stores. Readmission of external Ca\(^{2+}\), once Ca\(^{2+}\) release has terminated, results in a large rise in cytosolic Ca\(^{2+}\) due to Ca\(^{2+}\) entry through CRAC channels (Bird & Putney, 1993). We compared the rate of rise and extent of the cytosolic Ca\(^{2+}\) rise following Ca\(^{2+}\) entry through store-operated channels in intact cells in the presence or absence of extracellular Na\(^+\). To ensure intracellular Na\(^+\) was reduced following the removal of external Na\(^+\), we loaded cells with fura 2 in Na\(^+\)-free external solution and then maintained the

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**Figure 6. Activation of I\(_{\text{CRAC}}\) following extensive dialysis of the cytosol with Na\(^+\)-free pipette solution**

A, traces comparing time course of I\(_{\text{CRAC}}\) following dialysis with either Na\(^+\)-free external and pipette solutions (labelled 0 Na\(^+_i\), 0 Na\(^+_o\)) or Na\(^+\)-containing solutions (+Na\(^+_i\), + Na\(^+_o\)). Cells were kept in Na\(^+\)- and Ca\(^{2+}\)-free extracellular solution for ~10 min prior to break-in. Blue trace shows I\(_{\text{CRAC}}\) after dialysis with Na\(^+\)-free pipette solution for ~220 s before external Ca\(^{2+}\) was readmitted. Red trace is a control recording taken in the presence of Na\(^+\)-containing extracellular and pipette solutions. B, I–V curves, taken once the currents in panel A had peaked. C, amplitude of I\(_{\text{CRAC}}\) compared for the conditions shown. Each bar is the mean of between 6 and 9 cells. No significant difference was found between the 2 groups (\(P > 0.1\)). No significant difference in peak amplitude was seen with these various times of Ca\(^{2+}\) readmission (applied ~60-220 s after break-in) and all recordings for each condition have been combined.

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**Figure 7. Impact of removing extracellular Na\(^+\) on I\(_{\text{CRAC}}\) activated by Ins\(P_3\) in weak intracellular Ca\(^{2+}\) buffer and energized mitochondria**

A, time course of I\(_{\text{CRAC}}\) compared between a cell exposed to Na\(^+\)-containing external and pipette solutions (red trace) and a cell dialysed with Na\(^+\)-free solution and placed in Na\(^+\)-free external solution (16 min before break-in; blue trace). In both cases, pipette solution contained weak Ca\(^{2+}\) buffer, mitochondrial cocktail and Ins\(P_3\). Dialysis with a Na\(^+\)-containing pipette solution containing weak Ca\(^{2+}\) buffer and cocktail without Ins\(P_3\) failed to activate a current (black trace). B, I–V curves taken when the currents in panel A had reached steady state. C, the activation time constant compared for the different conditions. There were no statistically significant differences between the groups. D, bar chart plotting amplitude of I\(_{\text{CRAC}}\) for the conditions shown. Cocktail is mean of 7 cells, Na\(^+_o\)/Na\(^+_i\) 9 cells and 0 Na\(^+_o\)/0 Na\(^+_i\) 10 cells. There was no significant difference between Ins\(P_3\) groups. Both these groups were significantly different from cocktail alone (\(P < 0.01\) in each case). Cocktail solution without Ins\(P_3\) was +Na\(^+_o\); +Na\(^+_i\).
Figure 8. Effect of removing external Na\(^+\) on store-operated Ca\(^{2+}\) entry and cytosolic Ca\(^{2+}\) oscillations

A, application of the Na\(^+\) ionophore gramicidin A increases cytosolic Na\(^+\) when applied in Na\(^+\)-containing extracellular solution to CoroNa green-loaded cells (black trace, mean of 9 cells). The red trace (mean of 7 cells) shows the linear decrease in CoroNa green fluorescence in non-stimulated (control) cells. The black trace (mean of 7 cells) shows the change in cytosolic Na\(^+\) in cells loaded with dye in Na\(^+\)-containing solution but exposed to Na\(^+\)-free external solution just prior to gramicidin exposure. The red trace (mean of 10 cells) is the decay fluorescence in non-stimulated (control) cells.

B, black trace (mean of 10 cells) shows the change in cytosolic Na\(^+\) in cells loaded with dye in Na\(^+\)-free external solution and then maintained in Na\(^+\)-free external solution during gramicidin exposure. The red trace (mean of 9 cells) is the decay in fluorescence in non-stimulated (control) cells and superimposes on the black one.

D, bar chart comparing absolute CoroNa green fluorescence in control cells after loading in Na\(^+\)-containing external solution (black bar, mean of 9 cells) or after loading and then being kept in Na\(^+\)-free solution (red bar, mean of 9 cells). The y-axis denotes absolute fluorescence (AF).

E, store-operated Ca\(^{2+}\) influx is reduced in Na\(^+\)-free solution compared to Na\(^+\)-containing solution. The black trace (mean of 7 cells) shows the change in cytosolic Na\(^+\) in cells loaded with dye in Na\(^+\)-free solution but exposed to Na\(^+\)-containing external solution just prior to gramicidin exposure. The red trace (mean of 10 cells) is the decay fluorescence in non-stimulated (control) cells.

F, bar chart comparing absolute CoroNa green fluorescence in control cells after loading in Na\(^+\)-containing external solution (black bar, mean of 9 cells) or after loading and then being kept in Na\(^+\)-free solution (red bar, mean of 9 cells). The y-axis denotes absolute fluorescence (AF). E, store-operated Ca\(^{2+}\) entry is reduced in Na\(^+\)-free solution compared to Na\(^+\)-containing solution.
cells in Na⁺-free solution. Cells were therefore exposed continuously to Na⁺-free solution for >1 h prior to recording. We checked whether this protocol indeed lowered cytosolic Na⁺. To this end, cells were loaded with the Na⁺-sensitive fluorescent dye CoroNa green in either the presence or the absence of external Na⁺ and then subsequently challenged with the Na⁺ ionophore gramicidin A. Gramicidin A aggregates slowly in the plasma membrane to form Na⁺-permeable ion channels. In cells loaded with CoroNa green in the continuous presence of extracellular Na⁺, application of gramicidin A led to a prominent rise in cytosolic Na⁺ due to Na⁺ entry (Fig. 8A). No such increase was seen in control cells not exposed to gramicidin A (Fig. 8A). In cells that were loaded with CoroNa green in the presence of extracellular Na⁺ but were then exposed to Na⁺-free solution briefly (~2 min) before challenge with gramicidin A, no rise in cytosolic Na⁺ occurred (Fig. 8B). Cytosolic Na⁺ in fact declined somewhat in these cells after exposure to gramicidin A as cytosolic Na⁺ was initially higher than the Na⁺ concentration in Na⁺-free extracellular solution and therefore exited the cell through the gramicidin A channels in the plasma membrane (Fig. 8B). If cells were loaded with CoroNa green in Na⁺-free extracellular solution and then maintained in Na⁺-free solution, gramicidin A exposure failed to alter cytosolic Na⁺ (Fig. 8C), consistent with these cells having significantly reduced cytosolic Na⁺. We compared the absolute levels of CoroNa green intensity in cells loaded in Na⁺-containing external solution with those loaded in Na⁺-free solution. The absolute fluorescence was significantly lower in the latter cells (Fig. 8D). Therefore cytosolic Na⁺ was considerably reduced in cells exposed to Na⁺-free extracellular solution for >1 h, confirming that the use of Na⁺-free solution during the loading process was indeed effective.

In cells loaded with fura 2 in the continuous presence of extracellular Na⁺, stimulation with thapsigargin evoked Ca²⁺ release from the stores followed by prominent store-operated Ca²⁺ entry (Fig. 8E; aggregate data summarized in Fig. 8F). In cells loaded in Na⁺-free solution, a condition that lowers cytosolic Na⁺ (Fig. 8D), both Ca²⁺ release and store-operated Ca²⁺ entry were not significantly different from corresponding responses obtained in the presence of extracellular Na⁺ (Fig. 8E and F).

In some cell types, a rise in cytosolic Ca²⁺ activates non-selective TRPM4 leading to membrane depolarization (Vennekens et al. 2007; Holzmann et al. 2015). This reduces Ca²⁺ influx and constitutes a negative feedback mechanism for regulation of store-operated Ca²⁺ entry (Launay et al. 2004). Removal of extracellular Na⁺ would therefore be expected to enhance store-operated Ca²⁺ influx. However, we found that removal of Na⁺ had no effect on the rate or extent of Ca²⁺ influx (Fig. 8E and F). Two pieces of evidence argue against a role for TRPM4 in regulating CRAC channels under our conditions. First, dialysis of RBL cells with pipette solutions containing free Ca²⁺ in the range 300 nM to 2 μM, well within the range for robust activation of TRPM4 channels (Launay et al. 2002), failed to activate any current in the presence or absence of external Na⁺ (Bakowski and Parekh, unpublished data). Second, only Iᵥ,RAC develops following dialysis with a pipette solution containing InsP₃ and thapsigargin to deplete stores and 2 mM ATP as the only exogenous Ca²⁺ buffer (Fierro and Parekh, 2000). Under these conditions, free Ca²⁺ is weakly buffered at ~1 μM and will increase further upon CRAC channel activation. Nevertheless, only the Ca²⁺-selective CRAC current developed in the presence of external Na⁺.

Cytosolic Ca²⁺ oscillations are unaffected by removal of extracellular Na⁺

Physiological Ca²⁺ signals are often generated following stimulation of Gq-coupled receptors and are presented to cells in the form of repetitive cytosolic Ca²⁺ oscillations (Thomas et al. 1996). Activation of cysteinyl leukotriene type 1 receptors in RBL cells with the agonist leukotriene C₄ (LTC₄) evokes repetitive cytosolic Ca²⁺ oscillations, which arise from regenerative Ca²⁺ entry through CRAC channels (Di Capite et al. 2009). The Ca²⁺ entry component refills the stores (Di Capite et al. 2009) and repletes phosphatidylinositol 4,5-bisphosphate levels in readiness for the next oscillatory cycle (Alsiewid & Parekh, 2015). The Ca²⁺ oscillations run down quickly in the absence of external Ca²⁺, following knock down of Orai1 or after pharmacological block of CRAC channels (Kar et al. 2012a). If Na⁺ entry is required for sustaining
CRAC channel activity, a prediction would therefore be that Ca\(^{2+}\) oscillations evoked by leukotriene receptor stimulation should also run down quickly in Na\(^{+}\)-free external solution as CRAC channel activity should be compromised. To test this, we compared the amplitude and frequency of cytosolic Ca\(^{2+}\) oscillations generated in response to LTC\(_4\) stimulation in cells maintained either in the presence or in the absence of external Na\(^{+}\). Numerous Ca\(^{2+}\) oscillations were observed in the presence of extracellular Na\(^{+}\) (Fig. 8G), and the amplitude and number of oscillations gradually declined over time (Fig. 8H and I), due to receptor desensitization (Ng et al. 2012). In cells loaded and then maintained in Na\(^{+}\)-free solution for >1 h, LTC\(_4\) still elicited numerous Ca\(^{2+}\) oscillations (Fig. 8G) and the amplitude and number of oscillations were similar to those obtained in the presence of Na\(^{+}\) (Fig. 8H and I).

**CRAC channel-dependent gene expression is unaffected by removal of Na\(^{+}\)**

Ca\(^{2+}\) microdomains near open CRAC channels activate the transcription factors c-fos and NFAT1 through recruitment of local signalling pathways (Ng et al. 2009; Kar et al. 2014). Although \(I_{\text{CRAC}}\), store-operated Ca\(^{2+}\) entry and agonist-evoked physiological Ca\(^{2+}\) signalling were all unaffected by removal of external Na\(^{+}\) and the subsequent reduction in cytosolic Na\(^{+}\), we considered the possibility that cytosolic Na\(^{+}\) regulated CRAC channel-driven responses over longer time periods. To test this, we measured Ca\(^{2+}\)-dependent gene expression following CRAC channel activation. Stimulation with LTC\(_4\) for 10 min in the presence of external Na\(^{+}\) evoked a robust increase in the expression of c-fos (Fig. 9A). Exposure to Na\(^{+}\)-free solution for >1 h prior to challenge with LTC\(_4\) in Na\(^{+}\)-free solution also resulted in an increase in c-fos that was not significantly different in size from the response obtained in Na\(^{+}\)-containing solution (Fig. 9A; aggregate data are summarized in Fig. 9B). We also measured NFAT-driven gene expression using a GFP reporter gene under the control of an NFAT promoter (Kar et al. 2012b). Stimulation with LTC\(_4\) for 10 min in the presence of external Na\(^{+}\) resulted in GFP expression 24 h later (Fig. 9C and D). A similar level of reporter gene expression was obtained when cells were exposed to Na\(^{+}\)-free solution for >1 h before stimulation with LTC\(_4\) in Na\(^{+}\)-free solution (Fig. 9C and D). Identical results were obtained when we used a strong stimulus to maximally activate CRAC channels. Stimulation with thapsigargin activated c-fos (Fig. 9E and F) and NFAT reporter gene expression (Fig. 9G and H) both in the presence and in the absence of external Na\(^{+}\), and to similar extents.

**MCU is required for \(I_{\text{CRAC}}\) development under physiological conditions**

Mitochondrial Ca\(^{2+}\) uptake is necessary for the development of \(I_{\text{CRAC}}\) under physiological conditions of weak intracellular Ca\(^{2+}\) buffering. To examine whether the MCU channel was involved, we compared the size of the current in control cells with that in cells in which the MCU had been knocked down using a siRNA-based strategy. MCU knockdown reduced the rate of Ca\(^{2+}\) entry following readmission of external Ca\(^{2+}\) to thapsigargin-treated cells by 71 ± 5%, consistent with our previous study using the same siRNA construct (Samanta et al. 2014). Dialysis with InsP\(_3\) in weak Ca\(^{2+}\) buffer supplemented with mitochondrial cocktail evoked a clear \(I_{\text{CRAC}}\) in control cells (Fig. 10A; \(I–V\) relationship is shown in Fig. 10B and mean amplitude in Fig. 10C). However, the current was significantly smaller following knockdown of the MCU (Fig. 10A and C). If the role of the MCU under these conditions is to enable mitochondrial Ca\(^{2+}\) uptake, then one would expect this function to be obviated by dialysis with strong intracellular Ca\(^{2+}\) buffer as the latter would capture the vast majority of cytosolic Ca\(^{2+}\) and thereby render a role for mitochondrial Ca\(^{2+}\) uptake obsolete. Consistent with this, dialysis with a pipette solution containing InsP\(_3\), mitochondrial cocktail and strong Ca\(^{2+}\) buffer (10 mM EGTA) activated robust \(I_{\text{CRAC}}\) (Fig. 10D and F) that was unaffected by MCU knockdown with regards to time course of development, \(I–V\) relationship and peak amplitude (Fig. 10D–F).

**Effect of NCLX knockdown on \(I_{\text{CRAC}}\)**

To assess the role of mitochondrial NCLX on \(I_{\text{CRAC}}\) activation, we used an siRNA-based approach to knock down expression of the transporter. Western blot analysis showed NCLX knockdown was 61 ± 6%, similar to what we had observed previously in RBL cells using the same siRNA construct (Samanta et al. 2018). We activated \(I_{\text{CRAC}}\) in a similar way to that used by Ben-Kassus Nissim et al. (2017), namely through passive depletions of stores by dialysis with strong buffer. Robust \(I_{\text{CRAC}}\) developed following dialysis with 10 mM EGTA in control recordings (Fig. 11A and B), to reach an amplitude (Fig. 11C) similar to that seen in Fig. 4. Knockdown of NCLX protein did not prevent the development of \(I_{\text{CRAC}}\), nor did it reduce the size of the current (Fig. 11A–C). In fact, there was a tendency for the amplitude of the current to increase in NCLX-deficient cells, although this was not statistically significant (Fig. 11C). We repeated these experiments but now under more physiological conditions by using a pipette solution that contained weak Ca\(^{2+}\) buffer and mitochondrial cocktail together with InsP\(_3\) to deplete the stores. \(I_{\text{CRAC}}\) activated in both control cells and in cells in
Figure 9. CRAC channel-dependent gene expression is unaffected by removing external Na\(^+\)

A, C-fos expression compared between cells stimulated with LTC\(_4\) (160 nM) in Na\(^+\)-containing external solution and cells stimulated in Na\(^+\)-free solution. B, aggregate data compared from 3 independent experiments. For stimulation in Na\(^+\)-free solution, cells were exposed to Na\(^+\)-free solution for 1 h and then kept in this solution during and after stimulation. C, images comparing NFAT–GFP reporter gene expression at rest and then after 10 min stimulation with LTC\(_4\) in the presence or absence of external Na\(^+\). D, aggregate data from experiments as in panel C compared. Cells were kept in Na\(^+\)-free solution for 1 h prior to LTC\(_4\) challenge and then maintained in Na\(^+\)-free solution both during stimulation and then after stimulation for a further 30 min before cells were...
which NCLX had been knocked down (Fig. 11D–F). As was the case with strong buffer (Fig. 11C), the current amplitude tended to be larger in NCLX knockdown cells (Fig. 11F), although this was not significant ($P = 0.1$). Knockdown of NCLX did not compromise the development of $I_{\text{CRAC}}$ in either strong or weak intracellular $\text{Ca}^{2+}$ buffer.

**Discussion**

Since the discoveries of STIM1 and Orai1, growing evidence attests to a central role for CRAC channels in controlling a plethora of spatially and temporally distinct signalling pathways (Parekh, 2010; Prakriya & Lewis, 2015). An understanding of the molecular mechanisms that regulate the channels will provide new insights into the function of store-operated $\text{Ca}^{2+}$ entry in a physiological context. Our results reveal that $\text{Ca}^{2+}$ uptake by the MCU channel is required for the full development of $I_{\text{CRAC}}$ under physiological conditions of intracellular $\text{Ca}^{2+}$ buffering. We also find that the development of $I_{\text{CRAC}}$ under a range of different conditions, of store-operated $\text{Ca}^{2+}$ entry, of cytosolic $\text{Ca}^{2+}$ oscillations to a physiological trigger as well as CRAC channel-dependent gene expression are all insensitive to changes in extracellular and intracellular $\text{Na}^+$. 

**Central role for the MCU channel**

Although dialysis with InsP$_3$ activates $I_{\text{CRAC}}$ in the presence of strong intracellular $\text{Ca}^{2+}$ buffer (10 mM EGTA or BAPTA) in whole-cell patch clamp recordings from RBL mast cells, the current does not develop when weak, physiological levels of buffer (0.1–0.35 mM) are used instead (Glitsch & Parekh, 2000). However, the current develops under these conditions when precautions are taken to ensure mitochondria are retained in an energized state by inclusion of a mitochondrial cocktail.
that maintains flux through the Krebs cycle (Gilabert & Parekh, 2000). The hyperpolarized potential across the inner mitochondrial membrane provides a large driving force for cytosolic Ca\(^{2+}\) entry into the matrix through the MCU channel, enabling more extensive store depletion in response to Ins\(_P_3\) challenge as well as reducing cytosolic Ca\(^{2+}\)-dependent slow inactivation of the CRAC channels in RBL cells (Gilabert & Parekh, 2000; Glitsch et al. 2002a). This combination results in robust \(I_{\text{CRAC}}\) development. The MCU complex is composed of the pore-forming MCU protein in combination with the essential subunit EMRE, MCUb and regulators MICU1 and MICU2 (Sancak et al. 2013; Mammucari et al. 2018). Our data demonstrate that knock-down of the MCU protein inhibits the development of \(I_{\text{CRAC}}\) under physiological conditions of weak intracellular Ca\(^{2+}\) buffering. However, \(I_{\text{CRAC}}\) develops fully in cells in which MCU has been knocked down when strong Ca\(^{2+}\) buffer is used, revealing that the role of MCU indeed involves mitochondrial Ca\(^{2+}\) uptake and therefore buffering of cytosolic Ca\(^{2+}\). This central role for the pore-forming MCU subunit in supporting \(I_{\text{CRAC}}\) under physiological conditions helps explain why cytosolic Ca\(^{2+}\) oscillations evoked by receptor stimulation run down quickly in MCU-deficient cells (Samanta et al. 2014). The loss of mitochondrial Ca\(^{2+}\) buffering accelerates Ca\(^{2+}\)-dependent slow inactivation of CRAC channels and thereby compromises store refilling, needed to maintain the oscillatory Ca\(^{2+}\) response. Mitochondrial Ca\(^{2+}\) uptake is essential for supporting CRAC channel activity under physiological conditions in other cell types including T cells (Hoth et al. 1997; Hoth et al. 2000; Quintana et al. 2007) and endothelia (Malli et al. 2003) where it is likely, in light of our data, to also require the MCU channel.

**CRAC channels do not require extracellular or internal Na\(^+\) for activation**

The unitary conductance of the CRAC channel is tiny, in the range of a few femtosiemens (Zweifach & Lewis, 1993), and is well beyond the bandwidth of current patch clamp amplifiers. Selectivity and gating properties of the channels perforce have been derived from analysis of the whole-cell current. Pioneering work on the biophysical properties of \(I_{\text{CRAC}}\) in mast cells, RBL cells, T lymphocytes and Jurkat T cells established several common features.

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**Figure 11. Effect of knockdown of NCLX protein on \(I_{\text{CRAC}}\) activation**

- **A**, time course of \(I_{\text{CRAC}}\) compared between a control cell and one in which NCLX was knocked down. Cells were dialysed with a pipette solution containing 10 mM EGTA to deplete stores passively.
- **B**, \(I-V\) curves, taken from panel A at steady state, compared.
- **C**, bar chart comparing amplitude of \(I_{\text{CRAC}}\) for the conditions shown (each bar is mean of 6 cells).
- **D**, time course of \(I_{\text{CRAC}}\) shown for a control cell and for one in which NCLX had been knocked down. Pipette solution contained weak Ca\(^{2+}\) buffer, mitochondrial cocktail and Ins\(_P_3\).
- **E**, \(I-V\) curves, taken from panel D at steady state.
- **F**, bar chart comparing amplitude of \(I_{\text{CRAC}}\) for the conditions shown (each bar is mean of 7 cells).
including high selectivity for Ca\textsuperscript{2+} over other cations including Na\textsuperscript{+}, steep inward rectification, a very positive reversal potential (\(>+80\) mV) and a very low unitary chord conductance (reviewed in Parekh & Penner, 1997). In fact, the selectivity of CRAC channels for Ca\textsuperscript{2+} over Na\textsuperscript{+} in various cell types has been found to be \(>1000:1\) (Hoth, 1995), at least as high as that exhibited by voltage-gated Ca\textsuperscript{2+} channels. These biophysical studies have provided an essential rulebook in assessing whether candidate genes encode the CRAC channel and played a central role in establishing that Orai1 comprised the CRAC channel pore (Prakriya & Lewis, 2015).

Recently, the unique Ca\textsuperscript{2+} selectivity of the CRAC channel has been questioned by the finding that store-operated Na\textsuperscript{+} entry is required for the development of \(I_{\text{CRAC}}\) (Ben-Kassus Nissim et al. 2017). In that study, removal of external Na\textsuperscript{+} led to a substantial reduction in the size of \(I_{\text{CRAC}}\) in RBL cells by \(\sim70\%\) and abolished the endogenous current completely in HEK293 cells. It was suggested that store-operated Na\textsuperscript{+} entry was essential for the full development of \(I_{\text{CRAC}}\) because the Na\textsuperscript{+} entry raised cytosolic Na\textsuperscript{+}, which was then taken into the mitochondria in exchange for matrix Ca\textsuperscript{2+} through the mitochondrial NCLX transporter. Removal of matrix Ca\textsuperscript{2+} apparently prevented production of mitochondrial reactive oxygen species (ROS) and mitochondrial
derived ROS was reported to inhibit CRAC channels by oxidizing Cys195 on the extracellular side of the channels in the plasma membrane. The molecular basis of the store-operated Na\textsuperscript{+} flux was unclear but was suggested to reflect permeation through store-operated Na\textsuperscript{+}-permeable TRP channels, which would have to be activated by store depletion simultaneously with CRAC channels (Ben-Kassus Nissim et al. 2017). Alternatively, the Na\textsuperscript{+} flux could reflect permeation through Orai1 protein. Regardless of the precise mechanism, the reported extracellular store-operated Na\textsuperscript{+} flux contemporaneous with, and essential for, \(I_{\text{CRAC}}\) activation would mean that whole-cell \(I_{\text{CRAC}}\) recordings have a Na\textsuperscript{+} component, challenging earlier interpretations of selectivity.

If the whole-cell store-operated current arose from two parallel currents, composed of \(I_{\text{CRAC}}\) and a Na\textsuperscript{+}-permeable current, then in addition to amplitude and time course of the current, key biophysical features such as the extent of inward rectification, the reversal potential of the current and the rate and extent of Ca\textsuperscript{2+}-dependent fast inactivation should be different in the absence of external Na\textsuperscript{+}. Our new data, based on systematic removal of external Na\textsuperscript{+}, internal Na\textsuperscript{+} or both, demonstrate that neither extracellular nor intracellular Na\textsuperscript{+} has any effect on the size of \(I_{\text{CRAC}}\), the extent of inward rectification, the time constant of activation, the reversal potential of the current or Ca\textsuperscript{2+}-dependent inactivation in RBL cells. In a human salivary gland cell line, a detailed study has revealed that Ca\textsuperscript{2+} entry through CRAC channels leads to insertion of TRPC1 channels into the plasma membrane (Cheng et al. 2011). \(I_{\text{CRAC}}\) activates first and this is followed by dramatic changes in the whole-cell current as inserted TRPC1 channels then contribute. These changes include (i) an increase in the size of the whole-cell current, (ii) a shift in the \(I-V\) relationship of the whole-cell current to show much less inward rectification, and (iii) a large leftward shift of \(\sim80\) mV in the reversal potential of the current. These changes were not seen in our experiments following alterations in extracellular Na\textsuperscript{+}, consistent with the absence of a Na\textsuperscript{+}-permeable current.

Removal of extracellular Na\textsuperscript{+} failed to affect any of the properties of \(I_{\text{CRAC}}\) that we have measured using InsP\textsubscript{3} or passive store depletion (high EGTA or thapsigargin) to activate the current either in strong or weak Ca\textsuperscript{2+} buffer. The simplest explanation of our data is that \(I_{\text{CRAC}}\) is a Ca\textsuperscript{2+}-selective current and its activation and maintenance in RBL cells does not require a parallel Na\textsuperscript{+} current across the plasma membrane. We considered the possibility that a Na\textsuperscript{+} current was essential for CRAC channel activation as reported but was so small that it failed to impact on any of the hallmarks of \(I_{\text{CRAC}}\) that we have measured. Calculations suggest this is very unlikely. The NCLX has a \(K_M\) for cytosolic Na\textsuperscript{+} of \(\sim10\) mM (Palty et al. 2010). In our experiments on RBL cells and in those reported in HEK cells (Ben-Kassus Nissim et al. 2017), \(I_{\text{CRAC}}\) was activated by passive store depletion using a Na\textsuperscript{+}-free pipette solution and our \(^{23}\)Na NMR analysis confirmed we indeed used Na\textsuperscript{+}-free solution. As the cytosol was extensively dialysed before \(I_{\text{CRAC}}\) developed, cytosolic Na\textsuperscript{+} would have been very low in our experiments. For a store-operated Na\textsuperscript{+} current to develop in parallel with \(I_{\text{CRAC}}\) and raise cytosolic Na\textsuperscript{+} rapidly within a few seconds to at least 10 mM, which would be required to enable effective mitochondrial NCLX activity, a whole-cell Na\textsuperscript{+} current of \(\sim100\) pA would be required (ignoring Na\textsuperscript{+} clearance by pumps). This is larger than the typical \(\sim50\) pA \(I_{\text{CRAC}}\) we have recorded in RBL cells and considerably larger than the \(\sim1\) pA store-operated current reported from HEK cells (Ben-Kassus Nissim et al. 2017).

Removal of external Na\textsuperscript{+} also had no effect on the cytosolic Ca\textsuperscript{2+} rise due to store-operated Ca\textsuperscript{2+} entry, on cytosolic Ca\textsuperscript{2+} oscillations that require Ca\textsuperscript{2+} entry through CRAC channels or on CRAC channel-dependent gene expression. In our patch clamp experiments, recordings were made after 15–40 min of continuous exposure to Na\textsuperscript{+}-free solution and the Ca\textsuperscript{2+} measurements were made after cells had been exposed to Na\textsuperscript{+}-free solution for up to 90 min. Our experiments with gramicidin A and CoroNa green confirmed that such protocols had lowered cytosolic Na\textsuperscript{+} considerably.
Interestingly, whole-cell \( I_{\text{CRAC}} \) has also been measured over several minutes in Jurkat T cells using a pipette solution totally devoid of Na\(^+\) (Zweifach & Lewis, 1995a,b). These cells are substantially smaller than RBL cells and whole-cell dialysis of Na\(^+\) will therefore be quick. The CRAC current developed normally in the T cells and showed little sign of inactivation, being maintained for tens of seconds in high intracellular Ca\(^{2+}\) buffer despite the absence of Na\(^+\) from the pipette solution. Moreover, noise analysis studies on CRAC channels were conducted in isotonic Ca\(^{2+}\)-containing extracellular solution lacking Na\(^+\) and with Na\(^+\)-free internal solution (Zweifach & Lewis, 1993). These recordings of \( I_{\text{CRAC}} \) from jurkat T lymphocytes in the absence of extracellular and intracellular Na\(^+\) are consistent with the detailed biophysical analysis presented here for RBL cells.

### Implications for the involvement of mitochondrial Ca\(^{2+}\) transporters in regulating \( I_{\text{CRAC}} \) in RBL cells

Our patch clamp data obtained under physiological conditions of weak intracellular Ca\(^{2+}\) buffering, active SERCA pumps and energized mitochondria demonstrate that Ca\(^{2+}\) uptake by the MCU channel is required for full development of \( I_{\text{CRAC}} \). These data are in good agreement with previous studies that established mitochondrial Ca\(^{2+}\) buffering sustained store-operated Ca\(^{2+}\) entry (Gilabert & Parekh, 2000; Hoth et al. 2000; Glitsch et al. 2002b) and we now show that the MCU is the route for mitochondrial Ca\(^{2+}\) uptake. The electrogenic mitochondrial NCLX protein is another important component of the Ca\(^{2+}\) handling machinery in the inner mitochondrial membrane which, when operating in forward mode, extrudes Ca\(^{2+}\) from the matrix in exchange for cytosolic Na\(^+\) (Palty et al. 2010). In depolarized mitochondria, NCLX can reverse and extrude matrix Na\(^+\) in exchange for cytosolic Ca\(^{2+}\) (Samanta et al. 2018). Because we took precautions to energize mitochondria, reverse mode NCLX activity does not contribute to mitochondrial Ca\(^{2+}\) uptake in our experiments.

NCLX operating in forward mode has been proposed to play an indispensable role in the development of \( I_{\text{CRAC}} \), taking up cytosolic Na\(^+\) that has been elevated by store-operated Na\(^+\) entry in matrix Ca\(^{2+}\) (Ben-Kassus Nissim et al. 2017). Our findings suggest that development of \( I_{\text{CRAC}} \) in RBL cells in the presence of weak intracellular Ca\(^{2+}\) buffering depends on Ca\(^{2+}\) uptake by the MCU, that the current develops normally in the absence of both extracellular and intracellular Na\(^+\) and that \( I_{\text{CRAC}} \) develops normally after knockdown of NCLX demonstrate that Na\(^+\)-dependent fluxes across the plasma membrane and mitochondria are not essential for supporting \( I_{\text{CRAC}} \) at least in RBL cells. Mitochondrial Ca\(^{2+}\) buffering capacity is high with an estimated Ca\(^{2+}\) binding ratio of \(~4000\) (Babcock et al. 1997) and therefore \( \sim 40 \) times larger than the Ca\(^{2+}\) binding ratio of cytosol in most non-excitable cells (Neher, 1995) including RBL cells, which have a Ca\(^{2+}\) binding ratio of \( \sim 150 \) (Bakowski and Parekh, unpublished). Moreover, the major Ca\(^{2+}\) buffering system within the matrix is the formation of calcium phosphate complexes, with inorganic phosphate (\( P_{\text{i}} \)) being rapidly taken up into the matrix through \( \text{H}_2\text{PO}_4^- \rightarrow \text{H}^+ + \text{symport} \). The \( P_{\text{i}} \) effect on mitochondrial buffering has an \( EC_{50} \) of \( 0.096 \) mM (Wei et al. 2015). As we included \( 1 \) mM \( P_{\text{i}} \) in our mitochondrial cocktail, Ca\(^{2+}\) buffering within the matrix would have been maintained effectively in our experiments.

We have previously demonstrated using electron microscopy that mitochondria in RBL cells are located away from the plasma membrane, with very few within \( \sim 100 \) nm of the cell periphery and most congregated \( ~500-1000 \) nm away (Singaravelu et al. 2011). Therefore mitochondria buffer bulk cytosolic Ca\(^{2+}\) in RBL cells rather than local Ca\(^{2+}\) entry near the CRAC channels. Consistent with this, neither the rate nor extent of Ca\(^{2+}\)-dependent fast inactivation of CRAC channels, a process driven by Ca\(^{2+}\) microdomains that extend \(< 10 \) nm from the channel pore (Zweifach & Lewis, 1995a), was affected by suppression of mitochondrial Ca\(^{2+}\) uptake (Gilabert & Parekh, 2000). The spatial segregation of mitochondria from CRAC channels at the plasma membrane explains why high concentrations of the slow Ca\(^{2+}\) chelator EGTA circumvent the need for the MCU channel in the activation of the current. It is important to stress that our findings are focused on RBL cells and we cannot rule out that mitochondrial NCLX plays an important role in supporting the slow store-operated current in HEK293 cells as reported (Ben-Kassus Nissim et al. 2017), if mitochondria are close to the Ca\(^{2+}\) channels in this cell type so they can compete effectively with exogenous Ca\(^{2+}\) buffer. Ongoing work in our laboratory is addressing this.

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Additional information

Competing interests

None declared.

Author contributions

All electrophysiological studies, fluorescence measurements and siRNA knockdown were carried out in the Department of Physiology, Anatomy and Genetics. NMR experiments were conducted in the Department of Chemistry. All authors carried out the experiments for which the data are shown, and analysed and interpreted the data. All authors contributed to the writing of this study. All authors have read and approved the final version of this manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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