



How Many Orai's Does It Take to Make a CRAC Channel?

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CRAC (Calcium Release-Activated Calcium) channels represent the primary pathway for so-called “store-operated calcium entry” – the cellular entry of calcium induced by depletion of intracellular calcium stores. These channels play a key role in diverse cellular activities, most noticeably in the differentiation and activation of T cells, and in the response of mast cells to inflammatory signals. CRAC channels are formed by members of the recently discovered Orai protein family, with previous studies indicating that the functional channel is formed by a tetramer of Orai subunits. However, a recent report has shown that crystals obtained from the purified *Drosophila* Orai protein display a hexameric channel structure. Here, by comparing the biophysical properties of concatenated hexameric and tetrameric human Orai1 channels expressed in HEK293 cells, we show that the tetrameric channel displays the highly calcium-selective conductance properties consistent with endogenous CRAC channels, whilst the hexameric construct forms an essentially non-selective cation channel.

The concept of a pathway for Ca^{2+} entry that was activated upon depletion of internal Ca^{2+} stores, specifically the endoplasmic reticulum, was first proposed by Putney some 25 years ago¹, and the biophysical properties of the underlying calcium release-activated calcium channels (CRAC channels) providing such an entry pathway were characterized a few years later^{2–5}. However, the molecular identity of the CRAC channel was only revealed relatively recently^{6–8}. As a result, it is now clear that the store-operated CRAC channels are formed by members of the Orai protein family. Although only one member of this family is present in non-vertebrates, three members are expressed in mammals (Orai1–3), and it is now clear that the endogenous CRAC channels in mammals are formed by a homomeric assembly of Orai1 proteins. As to the actual stoichiometric assembly of these proteins to form the functional channel, examination of various concatenated assemblies of different numbers of wild-type Orai1 subunits led us to propose that the CRAC channel was formed as a tetramer of Orai1 subunits⁹. Subsequently, this conformation was confirmed by other groups using a variety of different approaches including single-molecule photobleaching^{10,11} or brightness analysis¹², and high-resolution electron microscopic examination of purified Orai proteins¹³, although debate remains regarding whether functional tetramers only form following activation-induced dimerization of resting dimers^{11,14}. Recently, the proposed tetrameric stoichiometry of the functional CRAC channel has been challenged by a study examining the crystal structure formed by purified *Drosophila* Orai proteins¹⁵, where the authors concluded that the Orai subunits assembled as a hexamer. This proposed stoichiometry was further supported by cross-linking and size-exclusion chromatography, using partial sequences of the *Drosophila* Orai, specifically lacking most of the cytosolic N-terminus (119–351, and 132–341, respectively). Similar studies on the structure of crystals formed by purified proteins of other channel types, especially various K^+ channel subtypes^{16,17}, have shown that this approach can reveal important details on the structural elements determining the conductance and selectivity properties of the channel in question. However, as is common in such studies, successful crystal formation involved making certain deletions and mutations in the native Orai protein sequence. Given this, it would seem to be critical to confirm that the conductance properties of channels formed using this approach display features consistent with the native channel in question. In this context, Hou *et al.*¹⁵ incorporated a point mutation (V174A, equivalent to V102A in Orai1) into their expressed protein that enables constitutive activity in the absence of STIM¹⁸. Incorporation of the purified mutant protein into liposomes enabled them to demonstrate a clear flux of sodium ions in the absence of external divalent cations which was inhibited by Gd^{3+} , and was absent in experiments using purified Orai proteins bearing the K163W mutation (equivalent to the R91W mutation in Orai1).

SUBJECT AREAS:

CALCIUM SIGNALLING

ION CHANNEL SIGNALLING

PERMEATION AND TRANSPORT

PATCH CLAMP

Received

29 January 2013

Accepted

23 May 2013

Published

7 June 2013

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Results

Given the above contradictory findings, we chose to directly examine the functional properties of hexameric Orai1 channels in more detail. For this we generated a construct consisting of a concatenated hexameric assembly of full-length (i.e. unmutated) Orai1 subunits, and expressed this in HEK cells stably expressing STIM1. La^{3+} -sensitive store-operated currents in these cells were evaluated in standard whole-cell patch clamp recordings using a Ca^{2+} -free, Cs^+ -based (140 mM), internal (patch pipette) solution containing the potent InsP_3 -receptor agonist adenophostin A (2 μM). Under these conditions, maximal activation of store-operated Ca^{2+} currents occurred within approximately 85–120 s after achieving whole-cell conditions (see Supplementary Fig. S1), consistent with previous studies involving either Orai1 monomers, or concatenated Orai1 tetramers^{9,19}. Examination of the resulting store-operated conductance measured in a $\text{Na}^+/\text{Ca}^{2+}$ based external solution ($[\text{Na}^+] = 140 \text{ mM}$, $[\text{Ca}^{2+}] = 10 \text{ mM}$) revealed a current-voltage relationship showing inward rectification, with clear outward currents observed at voltages above the reversal potential of +20 mV (Fig. 1a). Such outward currents are not seen with endogenous CRAC channel currents^{3,20}, suggesting the presence of a significant permeability of the internal cation (Cs^+). We next examined the effect of reducing extracellular Ca^{2+} from 10 mM to 0.1 mM, a concentration that is sufficient to render Ca^{2+} currents through CRAC channels to negligible levels, but is still sufficient, along with the presence of external Mg^{2+} (1.2 mM), to prevent any development of divalent-free currents^{3,21–24}. This procedure resulted in only a modest reduction (less than 25%) in the inward current magnitude measured at -80 mV , and shifted the reversal potential to 0 mV (Fig. 1a). Together, these features are consistent with the presence of a significant permeability to the external Na^+ ions, and only a modest overall contribution of Ca^{2+} ion flux. In addition, a significant (2-to-3-fold) increase in the outward currents recorded at positive potentials was seen, indicating that the reduction in extracellular Ca^{2+} concentration resulted in an increased permeability of the channel to Cs^+ ions.

As a comparison, we performed the same experiments in the STIM1-stable HEK cells expressing a concatenated tetrameric assembly of Orai1 subunits. Here, store-operated currents obtained with the normal extracellular solution ($[\text{Na}^+] = 140 \text{ mM}$, $[\text{Ca}^{2+}] = 10 \text{ mM}$) showed strong inward-rectification with no detectible outward currents at voltages up to +80 mV (Fig. 1b). Moreover, reducing the extracellular Ca^{2+} concentration from 10 mM to 0.1 mM virtually eliminated all currents (inward currents at -80 mV were less than 10% of those recorded in extracellular media containing 10 mM Ca^{2+}). Clearly, the characteristics of the current obtained upon expression of the tetrameric Orai1 construct differ markedly from those seen with the hexameric construct. More importantly, the properties of the current displayed by the tetramer closely match those reported for endogenous CRAC channels^{2–5}, as well as those seen on simple overexpression of monomeric Orai1 and STIM1^{25–27}.

A recent study from the Prakriya group¹⁸ has indicated that the calcium selectivity of the channels formed by expression of Orai1 (either the wild-type Orai1, or the V102 mutant equivalent to that used in the study by Hou et al.¹⁵) can be markedly influenced, in a dose-dependent manner, by the level of co-expressed STIM1. This raises the possibility that the apparent lack of significant calcium-selectivity shown by the expressed concatenated hexamer might result from inadequate levels of cellular STIM1. To examine this, we transiently over-expressed STIM1 in our cells that were already stably expressing STIM1. Examination of the current-voltage relationship of the store-operated currents in these cells compared to those seen in the STIM1-stable cells without STIM1 overexpression and measured in the same $\text{Na}^+/\text{Ca}^{2+}$ based external solution ($[\text{Na}^+] = 140 \text{ mM}$, $[\text{Ca}^{2+}] = 10 \text{ mM}$) used above (see Fig. 1a), revealed that the overexpression of STIM1 failed to significantly affect either the overall shape of the I/V curve, the inward current magnitude

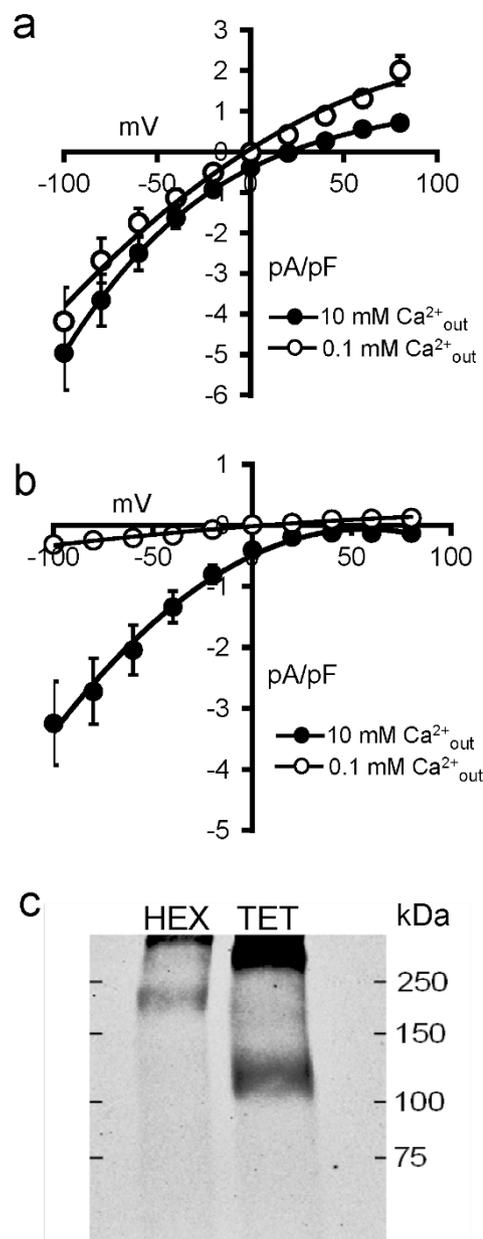


Figure 1 | Selectivity properties of expressed hexameric and tetrameric Orai1 concatamers. (a) Mean (\pm SE, $n = 6$) I/V curves for store-operated currents recorded between -100 mV and $+80 \text{ mV}$ in cells expressing the concatenated Orai1 hexamer in an external medium containing 140 mM Na^+ and either 10 mM Ca^{2+} , or 0.1 mM Ca^{2+} . (b) The corresponding mean (\pm SE, $n = 5$) I/V curves for store-operated currents in cells expressing the concatenated Orai1 tetramer. (c) Western blot showing the expressed FLAG-tagged concatamers running at their appropriate expected molecular masses (approximately 195 kDa for the hexamer, and 130 kDa for the tetramer).

measured at -80 mV ($P = 0.56$), the outward current measured at $+60 \text{ mV}$ ($P = 0.42$), or the reversal potential ($P = 0.42$) (see Supplementary Fig. S2). Consequently, we conclude that the relatively nonselective features of the expressed concatenated Orai1 hexamer were not a result of inadequate cellular STIM1 levels. In addition, unlike the endogenous CRAC channel^{3,28,29}, and the expressed Orai1 concatenated tetramer¹⁹, the expressed concatenated hexamer did not display any obvious Ca^{2+} -dependent fast-inactivation (CDI), even in the presence of 110 mM external calcium (Supplementary Fig. S3). Previous studies have shown that such a



loss of CDI in Orai1 channels can result from inadequate levels of cellular STIM1¹⁸. However, the demonstrated inability of overexpression of STIM1 in the STIM1-stable cells to affect the selectivity of the hexamer noted above, indicates that inadequate STIM1 is unlikely to be responsible for the absence of CDI.

The concatenated construct approach we have used is not without potential issues, particularly in relation to the correct processing of the expressed protein. However, the basic validity of this approach for Orai channels has been demonstrated in previous studies^{9,19,30}. Nevertheless, to evaluate the intact expression of the concatenated constructs used, we performed Western blot analysis of the concatenated hexamer and tetramer constructs (Fig. 1c). The data obtained clearly indicate that each of these constructs ran as a single band of the appropriate relevant molecular size.

To further examine the nature of the currents induced on expression of the hexameric Orai1 construct, we examined the effect of changing the major external cation from Na⁺ to NMDG⁺ ([NMDG⁺] = 140 mM, [Ca²⁺] = 10 mM). Store-operated currents recorded under these conditions displayed characteristics similar to those seen with the Na⁺-containing extracellular solution, namely an inwardly-rectifying current-voltage relationship with significant outward currents above the reversal potential of +20 mV. As noted above, this is indicative of the presence of a modest permeability to Cs⁺ ions (Fig. 2a). However, in marked contrast to the currents recorded in the Na⁺-based external solution, reducing extracellular

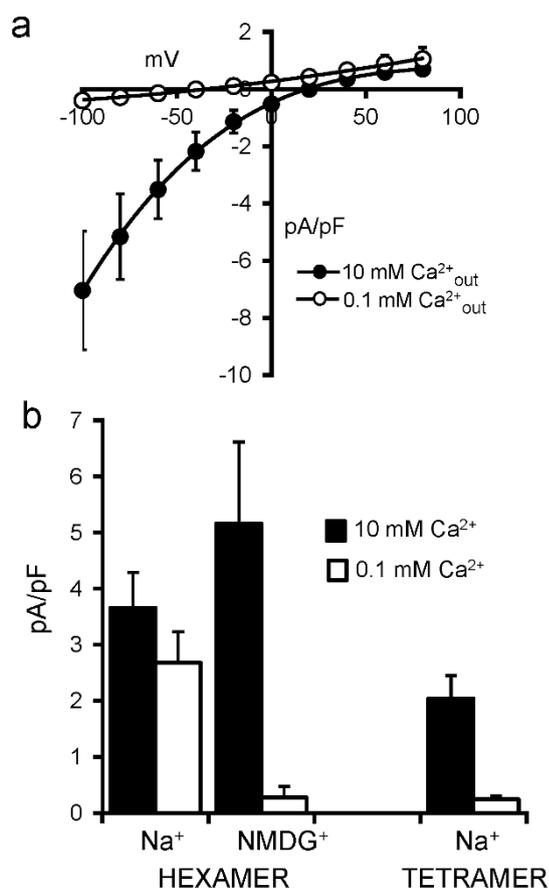


Figure 2 | The apparent selectivity properties of the expressed Orai hexamer are influenced by the major external cation. (a) Mean (\pm SE, $n = 5$) I/V curves for store-operated currents in cells expressing the concatenated Orai1 hexamer in an external medium containing 140 mM NMDG⁺ and either 10 mM Ca²⁺_{out} or 0.1 mM Ca²⁺_{out}. (b) Histogram comparing the effects of changing external calcium concentrations from 10 mM to 0.1 mM on inward currents measured at -80 mV, in cells expressing either the concatenated Orai1 hexamer or the Orai1 tetramer.

Ca²⁺ from 10 mM to 0.1 mM now virtually eliminated this current (greater than 90% reduction at -80 mV) (Fig. 2a). These results indicate that the inward current seen with NMDG⁺ as the extracellular monovalent cation predominantly reflects the inward flux of Ca²⁺ ions. The fundamental differences in conductance properties of the channels formed by these various constructs are summarized in Fig. 2b which compares the effect of changing the external Ca²⁺ concentration from 10 mM to 0.1 mM, on the magnitude of inward store-operated currents measured at -80 mV in cells expressing either the concatenated hexamer or the concatenated tetramer.

Discussion

Together, these results indicate that the concatenated hexamer forms a store-operated channel that, in addition to being permeable to Ca²⁺ ions, is also permeable to both Na⁺ and Cs⁺ (see Fig. 3), but not to NMDG⁺. This permeability to both Na⁺ and Cs⁺ in the presence of external Ca²⁺ stands in marked contrast to the highly Ca²⁺-selective properties of both the concatenated Orai1 tetramer⁹ and native CRAC channels under the same conditions, whether examined in mammalian cells^{3,31,32}, or in *Drosophila* cells^{7,33}. We can offer no explanation as to why the crystals obtained from the purified protein used by Hou *et al.*¹⁵ should have assembled to form what is a clearly organized hexameric structure – a finding supported by both cross-linking and size-exclusion chromatography. Of course, our approach involving expressed concatenated proteins differs markedly from that used by Hou *et al.*¹⁵. However, it should be noted that the selectivity properties of the hexameric channel, as assessed by Hou *et al.*¹⁵ – namely significant permeability to Na⁺ ions (even in the presence of divalent cations), but little or no permeability to NMDG⁺ – are entirely duplicated by our expressed concatenated Orai1 hexamer. Critically however, further examination of the selectivity properties of the concatenated hexamer revealed marked differences from those of the endogenous CRAC channels. Consequently, based on the data obtained, we conclude that the expression of a concatenated hexameric Orai1 channel results in an essentially non-selective cation channel whose biophysical “fingerprint” fails to replicate that of endogenous CRAC channels, or that seen on expression of a concatenated tetramer of Orai1.

Methods

The HEK293 cells stably expressing STIM1 were generated using the Flp-InTM-293 system (Invitrogen) as previously described⁹. Concatenated constructs comprising the appropriate number of intact Orai1 subunits were prepared as previously described⁹.

Cells were transiently transfected with 0.75 μ g of the Orai1 tetramer M070 or hexamer M070 along with 0.25 μ g YFP using an Amaxa Nucleofector. In some experiments, 0.5 μ g of STIM1pcmv6 was co-transfected with the Orai1 hexamer

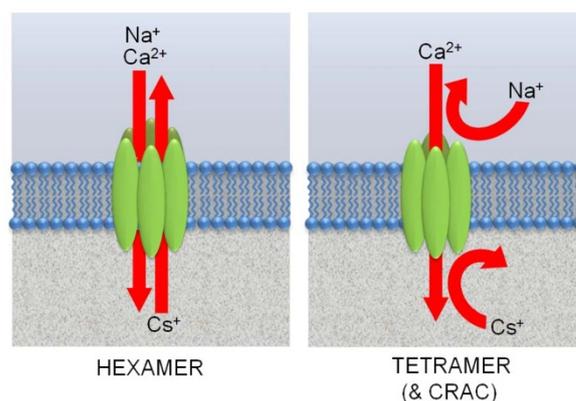


Figure 3 | Comparison of the different selectivity properties of the channels formed by the concatenated constructs. Diagram illustrating the distinct properties of the expressed Orai1 hexamer (left), and those of the expressed Orai1 tetramer, or endogenous CRAC channels (right).



M070. Transfected cells were incubated in EMEM + 10% FBS in a 5% CO₂ incubator for 40–48 hours before electrophysiological experiments were performed.

Electrophysiology. Patch clamp experiments were performed at room temperature (20–22°C) on cells 18–24 hours after plating on coverslips. Whole-cell current recordings were obtained using 250 ms voltage pulses to –80 mV delivered every 2 s from a holding potential of 0 mV. To obtain current-voltage relationships, 10 ms pulses to potentials between –100 mV and +80 mV were applied at 20 mV intervals. The standard internal (pipette) solution contained 140 mM CsC₂H₃O₂, 7.0 mM MgCl₂, 10 mM EGTA, and 10 mM Hepes (pH 7.2). Maximal activation of store-operated currents was achieved by inclusion of the potent InsP₃ receptor agonist adenophostin A (2 μM) in this Ca²⁺-free solution. In addition, the free Mg²⁺ concentration in the pipette solution was set at 5 mM to prevent any contamination from the so-called MIC or MagNum (TRPM7) currents^{32,34,35}. The standard extracellular solution contained 140 mM NaCl, 5 mM CsCl, 1.2 mM MgCl₂, 10 mM CaCl₂, 20 mM glucose, and 10 mM Hepes (pH 7.4). All currents were corrected by leak subtraction using currents obtained at the end of each experiment in an external solution containing La³⁺ (100 μM).

Western blot of concatenated Orai1 tetramers and hexamers. Cells stably expressing STIM1 were transfected with 2.5 μg of either the Orai1 tetramer or Orai1 hexamer in the M070 vector using Lipofectamine LTX (Invitrogen) for 48 hours. The cells were washed twice with ice-cold Tris buffered saline (TBS) and then lysed in 500 μl IP lysis buffer (Pierce) plus a “complete Mini protease inhibitor tablet” (Roche), transferred to an ice cold dounce homogenizer with a tight pestle and ground 25 times on ice. The samples were left on ice for 30 minutes. The lysate was then spun at 13000 g at 4°C for 10 minutes to spin down the nuclei and cell debris. 7.5 μl of a FLAG polyclonal antibody (Cell signaling #2368) was added to the supernatant and incubated at 4°C, rotating for 2 hours. 80 μl of lysis buffer prewashed A/G beads were added and rotated for 2 hours at 4°C, followed by washing five times with lysis buffer, and a final wash with TBS. The samples were run on a 7% SDS acrylamide gel. 2× SDS loading buffer plus DTT was added to the samples and heated to 80°C for 5 minutes. 20 μl of each sample was loaded on to the gel along with a Precision Plus dual color standard ladder (BioRad). Gels were transferred onto nitrocellulose in transfer buffer containing 25 mM Tris Base, 192 mM glycine, 10% methanol and 0.04% SDS in a wet transfer cassette at 23 mA constant current. The blot was probed with 1 : 1000 anti-FLAG polyclonal antibody (Cell Signaling #2368) followed by IRDye 800CW anti-rabbit secondary antibody at 1 : 10,000 dilution. Blots were analyzed on an Odyssey Scanner (Licor).

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Acknowledgements

We thank Dr. R. Dirksen for helpful comments on an early version of this manuscript. This study was supported National Institutes of Health grant GM040457 to TJS.

Author contributions

Both TJS and JLT contributed to the conception and analysis of the experiments, which were mainly carried out by JLT. TJS wrote the manuscript, with assistance from JLT.

Additional information

Supplementary information accompanies this paper at <http://www.nature.com/scientificreports>

Competing financial interests: The authors declare no competing financial interests.

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How to cite this article: Thompson, J.L. & Shuttleworth, T.J. How Many Orai's Does It Take to Make a CRAC Channel? *Sci. Rep.* **3**, 1961; DOI:10.1038/srep01961 (2013).