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Distinct pharmacological profiles of ORAI1, ORAI2, and ORAI3 channels

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Abstract

The ubiquitous Ca^{2+} release-activated Ca^{2+} (CRAC) channel is crucial to many physiological functions. Both gain and loss of CRAC function is linked to disease. While ORAI1 is a crucial subunit of CRAC channels, recent evidence suggests that ORAI2 and ORAI3 heteromerize with ORAI1 to form native CRAC channels. Furthermore, ORAI2 and ORAI3 can form CRAC channels independently of ORAI1, suggesting diverse native CRAC stoichiometries. Yet, most available CRAC modifiers are presumed to target ORAI1 with little knowledge of their effects on ORAI2/3 or heteromers of ORAIs. Here, we used ORAI1/2/3 triple-null cells to express individual ORAI1, ORAI2, ORAI3 or ORAI1/2/3 concatemers. We reveal that GSK-7975A and BTP2 essentially abrogate ORAI1 and ORAI2 activity while causing only a partial inhibition of ORAI3.

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The authors declare no competing interests.

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Interestingly, Synta66 abrogated ORAI1 channel function, while potentiating ORAI2 with no effect on ORAI3. CRAC channel activities mediated by concatenated ORAI1–1, ORAI1–2 and ORAI1–3 dimers were inhibited by Synta66, while ORAI2–3 dimers were unaffected. The CRAC enhancer IA65 significantly potentiated ORAI1 and ORAI1–1 activity with marginal effects on other ORAIs. Further, we characterized the profiles of individual ORAI isoforms in the presence of Gd^{3+} (5 μM), 2-APB (5 μM and 50 μM), as well as changes in intracellular and extracellular pH. Our data reveal unique pharmacological features of ORAI isoforms expressed in an ORAI-null background and provide new insights into ORAI isoform selectivity of widely used CRAC pharmacological compounds.

Graphical Abstract

	ORAI1	ORAI2	ORAI3
2-APB 5 μM 50 μM	$\uparrow$ $\downarrow$	$\uparrow$ $\downarrow$	- $\uparrow$
Gd^{3+} (5 μM)	$\downarrow$	$\downarrow$	$\downarrow$
BTP2 (10 μM)	$\downarrow$	$\downarrow$	$\downarrow$
GSK-7975A (10 μM)	$\downarrow$	$\downarrow$	$\downarrow$
Synta66 (10 μM)	$\downarrow$	$\uparrow$	-
IA65 (10 μM)	$\uparrow$	$\downarrow$	$\uparrow$
Acidic pH	$\downarrow$	$\downarrow$	-
Alkaline pH	$\uparrow$	$\uparrow$	-

$\uparrow/\uparrow$, slight/robust potentiation; $\downarrow/\downarrow$, slight/robust inhibition; -, marginal/no effect

Keywords

Calcium signaling; CRAC channels; ORAI1/2/3; pharmacological compounds; pH

Introduction

The store-operated Ca^{2+} entry (SOCE) pathway mediated by the Ca^{2+} release-activated Ca^{2+} (CRAC) channel is a ubiquitous receptor-activated Ca^{2+} entry route across the plasma membrane that is critical to many cellular functions in mammalian organisms[1–8]. CRAC channel function depends on two classes of membrane proteins: 1) The Ca^{2+} sensing stromal interaction molecule (STIM) located in the membrane of the endoplasmic reticulum (ER); and 2) the plasma membrane channel proteins, ORAIs[1–4]. Agonists acting on membrane receptors that couple to the phosphoinositide-specific phospholipase C (PLC) cause production of inositol-1,4,5-trisphosphate (IP_3) from the breakdown of phosphatidylinositol(4,5)bisphosphate (PIP_2)[9]. It is clearly established that the depletion of ER Ca^{2+} by the action of IP_3 on IP_3 receptors cause STIM1 proteins to translocate to ER-plasma membrane junctions where they trap ORAI1 channel protein to activate CRAC channels and induce SOCE[1, 2, 4, 10]. Both loss or gain of function mutations in STIM1 and ORAI1 have been linked to many diseases, including severe combined immune-deficiency (SCID), autoimmunity, ectodermal dysplasia, muscle weakness, tubular aggregate myopathy, and Miosis[6, 11]. Further, remodeling of STIM and ORAI protein expression

contributes to an increasing number of cardiovascular and respiratory diseases and cancers [12, 13], highlighting the potential therapeutic use of specific CRAC channel modifiers during disease [14].

Mammals express two STIM proteins (STIM1/2) and three ORAI proteins (ORAI1–3), which are encoded by five independent genes. There is strong evidence that mammalian CRAC channels are hexamers of ORAI proteins [15–17] and that genuine and optimal CRAC channel activation requires that each ORAI subunit within hexameric CRAC is bound to one STIM protein [18, 19]. While STIM1 and ORAI1 are the *bona fide* components of CRAC channels, increasing evidence suggest that ORAI2 and ORAI3 proteins heteromerize with ORAI1 under native conditions to fine tune CRAC channel activity [20–22]. STIM2, long considered as a mediator of homeostatic SOCE under unstimulated basal conditions, has recently been shown to recruit STIM1 to ORAI1 clusters to amplify CRAC channel activity under relatively weak agonist stimulation [23–25]. Furthermore, in some cell types such as neurons, monocytes and certain cancer cells, STIM2, ORAI2 and ORAI3 mediate a sizeable portion of native SOCE [26–32], suggesting that CRAC channels encoded by ORAI2 or ORAI3 independently of ORAI1 likely exist at least in some cell types, especially when ORAI1 expression is low or absent.

While there are currently no inhibitors or activators of CRAC channels that are in use in the clinic, several CRAC channel inhibitors have undergone clinical trials in recent years [14]. Current CRAC channel modifiers commercially available have been presumed to act on ORAI1 in native cell systems where other STIM/ORAI isoforms are present [33–42]. Furthermore, overexpression studies have focused on STIM1/ORAI1-mediated CRAC channel pharmacology in wildtype cells where these CRAC currents might be contaminated by endogenous STIM/ORAI isoforms [36–38, 40, 42]. Here, we used our ORAI1/2/3 triple knockout HEK293 (ORAI-TKO) cells developed recently [22], which provides an ORAI-null background, to co-express STIM1 with individual ORAI isoforms or combinations of ORAI concatenated dimers. We used whole cell patch clamp electrophysiology to determine the unique pharmacological features of CRAC currents (I_{CRAC}) mediated by different ORAIs. In addition to the established CRAC channel inhibitors 2-Aminoethoxydiphenyl borate (2-APB) [36, 38, 41, 42] and the trivalent ion Gd^{3+} [43, 44], we evaluated other CRAC channels inhibitors, the pyrazole derivatives BTP2 and GSK-7975A, Synta66 [38, 39] as well as a new CRAC enhancer IA65 characterized by our group [26]. Previous studies suggested that Ca^{2+} signaling in immune cells is remodeled within the inflammatory environment with upregulation of ORAI3. Unlike ORAI1, ORAI3 appears resistant to inhibition by the oxidant-rich inflammatory environment [32, 45]. We also studied the effects of intracellular and extracellular oxidizing and reducing environments on the activity of CRAC channels [46–49], while considering each ORAI isoform independently expressed in an ORAI-null background. Our results reveal unique pharmacological features of different ORAI isoforms and concatenated ORAI combinations and provide a comprehensive pharmacological guide for the identification of the molecular nature of CRAC channels in various native cell types and tissues with distinct patterns of ORAI isoform expression.

Materials and Methods

Reagents

2-aminoethoxydiphenyl borate (2-APB) (Catalog number: 100065–100MG), GSK-7975A (Catalog number: 534351), and Synta66 (Catalog number: SML1949–5MG) were purchased from Millipore-Sigma. BAPTA, Tetracesium Salt (Catalog number: B1212) was purchased from Invitrogen. BTP2 (catalog number: 3939) was purchased from TOCRIS. $GdCl_3$ was from Acros Organics. IA65 was recently described [26]. All other reagents used in this study were purchased from Thermo Fisher. The transfection kit for HEK293 cells (Catalog number: VCA-1003) was from Lonza.

Cell culture and transfections

HEK293 cells (ATCC) were transfected with 4.0 μg EYFP-STIM1 along with 1.0 μg of either CFP-ORAI1, CFP-ORAI2, CFP-ORAI3, or CFP-tagged concatenated ORAI dimers [15, 22] and seeded on 30 mm round glass coverslips in 6-well plates one day before patch-clamp recordings. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 1% l-glutamine, 1% sodium pyruvate, 10% fetal bovine serum (FBS), and 1% Antibiotic-Antimycotic (100X) (Thermo Fisher, catalog number: 15240062), at 37°C in a humidified atmosphere containing 5% CO_2 . Detailed methods on the generation of ORAI1/2/3 triple knockout HEK293 cells using CRISPR/Cas9 from ORAI1 single knockout cells were previously described [22, 26, 27, 50, 51].

Patch clamp electrophysiology

Whole-cell patch clamp electrophysiological recordings were performed with an Axopatch 200B and Digidata 1440A (Molecular Devices). Patch pipettes were pulled from borosilicate glass capillaries (World Precision Instruments, Sarasota, FL) with a P-1000 Flaming/Brown micropipette puller (Sutter Instrument) and polished with DMF1000 (World Precision Instruments). Resistances of filled patch pipettes were 2 to 4 M Ω . Under whole-cell configuration, only cells with small series resistances (< 8 M Ω) and tight seals (>16 G Ω) were chosen to perform recordings. A programmed voltage ramps from –140 mV to +100 mV (from a holding potential of +30 mV) was applied every 3 seconds. 8 mM $MgCl_2$ was included in the pipette solution to inhibit TRPM7 currents. Clampfit 11.1 software (Molecular Devices) was used to analyze data. Bath solution: 115 mM Na-methanesulfonate, 10 mM CsCl, 1.2 mM $MgSO_4$, 10 mM Hepes, 20 mM $CaCl_2$, and 10 mM glucose (pH adjusted to 7.4 with NaOH). Pipette solution: 115 mM Cs-methanesulfonate, 20 mM Cs-BAPTA, 8 mM $MgCl_2$, and 10 mM Hepes (pH adjusted to 7.2 with CsOH). For extracellular pH effects on I_{CRAC} : Bath solution: 115 mM Na-methanesulfonate, 10 mM CsCl, 1.2 mM $MgSO_4$, 10 mM Hepes, 20 mM $CaCl_2$, and 10 mM glucose (pH adjusted to 5.3, 7.4 and 9.5 with either HCl or NaOH). For intracellular pH effects on I_{CRAC} : Pipette solution: 115 mM Cs-methanesulfonate, 20 mM Cs-BAPTA, 8 mM $MgCl_2$, and 10 mM Hepes (pH adjusted to 5.0, 7.2 and 9.5 with either HCl or CsOH). For ORAI1- and ORAI2-mediated current recordings, we always subtract the first sweep immediately after break-in to correct for any contribution from leak currents. Because ORAI3 tend to generate pre-activated currents, we subtract the last sweep after block with 5 μM Gd^{3+} .

Statistical analysis

All values are presented as boxplots with the 25th to 75th percentile range, mean and median for each condition. Paired Sample t-Test or one-way ANOVA were used for two or three groups comparisons, respectively. Data were plotted using Origin software, with $p < 0.05$ considered significant.

Results and Discussion

To rule out confounding effects from endogenous ORAI isoforms, we used ORAI1/2/3 triple knockout HEK293 (ORAI-TKO) cells, in which we co-expressed STIM1 with various ORAI variants and recorded steady-state CRAC currents mediated by these ORAI variants followed by addition of CRAC channel inhibitors and potentiators. To minimize contributions from CRAC channel slow Ca^{2+} -dependent inactivation (slow CDI), we depleted the ER stores by inclusion of 20 mM of the fast Ca^{2+} chelator BAPTA in the patch pipette. Previous studies have showed that native CRAC channels are inhibited by low concentrations of trivalent lanthanides (1–5 μM Gd^{3+}) [26, 28, 29, 33, 35, 43, 44, 52]. While the trivalent lanthanides Gd^{3+} and La^{3+} are broad inhibitors of many ion channels and transporters, low concentrations of these trivalent ions ($<10 \mu\text{M}$) selectively block highly Ca^{2+} selective channels such as CRAC channels and L-type voltage-gated $\text{Ca}_v1.2$ Ca^{2+} channels by interfering with the selectivity filter within the channel pore [53–56], suggesting that Ca^{2+} selectivity and inhibition by low μM lanthanides go hand in hand. We show here that 5 μM Gd^{3+} equally and significantly blocks CRAC currents mediated by ORAI1, ORAI2 and ORAI3 (Fig. S1). These results are consistent with the fact that all ORAI isoforms mediate Ca^{2+} selective CRAC conductances and that the pore regions of all ORAIs are highly homologous [1].

2-APB is an extensively characterized pharmacological modulator of CRAC channels, which was shown to exhibit both a potentiating and inhibitory effect on native CRAC channel activity depending on the concentration used. Low concentration (1–10 μM) of 2-APB potentiated CRAC currents (I_{CRAC}), while high concentration (20–50 μM) of 2-APB inhibited I_{CRAC} [36, 40–42]. In studies of ORAI isoforms ectopically expressed in wildtype HEK293 cells, high concentrations of 2-APB potentiated ORAI3 and altered its ion selectivity [36, 38, 40, 42]. Here we show that 5 μM 2-APB potentiates I_{CRAC} mediated by ORAI1 and ORAI2 but has no effect on ORAI3 I_{CRAC} (Fig. S2). However, 50 μM 2-APB completely abrogates ORAI1, but only slightly inhibits ORAI2. Further, 50 μM 2-APB significantly potentiated ORAI3 and altered its CRAC current/voltage (I/V) relationship revealing outward rectification (Fig. S3). These results are consistent with previous findings showing that 2-APB causes a dilation of ORAI3 pore rendering the channel non-selective [57]. Our findings on ORAI isoform expression in ORAI-TKO cells show that ORAI1 is more sensitive to inhibition by 2-APB than ORAI2.

Most ion channels are regulated by changes of internal and external pH. Native CRAC channels and those mediated by ORAI isoforms expressed in wildtype HEK293 cells have been shown to be regulated by acidic or alkaline pH [46–49]. Intracellular (Fig. S4) or extracellular acidification (Fig. S5; pH=5.0–5.3) inhibited CRAC currents mediated by ORAI1 and ORAI2, but had no effect on ORAI3 I_{CRAC} , when compared to physiological

pH (pH=7.2). Conversely, intracellular (Fig. S4) and extracellular alkalization (Fig. S5; pH=9.5) potentiated ORAI1 and ORAI2, with marginal effect on ORAI3. Early studies, most of which predate the discovery of STIM and ORAI proteins have reported the regulation of native I_{CRAC} by both extracellular and intracellular pH in Jurkat T-cells, macrophages and neutrophils[58–61]. These reports showed that extracellular and intracellular acidification inhibits while alkalization enhances I_{CRAC} . I_{CRAC} generated through ectopic expression of ORAI1 (with STIM1) showed similar sensitivities to both internal and external low and high pH albeit with some subtle discrepancies, including those related to the pKa values of acidic pH block [46, 49]. These discrepancies are likely due to the notion that CRAC channels in native cell systems are contributed by homomeric and/or heteromeric assemblies of several ORAI isoforms[62]. Previous studies showed that several residues mediate the sensitivity of ORAI1 to external pH and these include D110, D112, E106 and E190[46, 48, 49]. Tsujikawa et al showed that H155 located in the TM2-TM3 intracellular loop mediates ORAI1 sensitivity to internal pH[49]. Interestingly, all these residues including H155 are conserved in ORAI3, suggesting that additional residues or pathways are mediating the resistance of ORAI3 to changes in pH. Oxidation of C195 of ORAI1 was shown to mediate its H_2O_2 -dependent inhibition [35, 45]. ORAI3 is resistant to H_2O_2 -dependent inhibition and the equivalent residue of C195 in ORAI3 is G170[45]. It is interesting to note that reactive oxygen species such as H_2O_2 are typically high in the tumor microenvironment which is also acidic[63]. The ability of ORAI3 to maintain activity in the tumor microenvironment or in inflammatory environments is likely important and deserves further attention [64]. Our results show that ORAI1 and ORAI2 are significantly inhibited by both intracellular and extracellular acidification and potentiated by alkalization, while ORAI3 is only marginally affected. The molecular determinants of these differences in pH sensitivity between ORAI3 and ORAI1/2 and whether G170 contributes to ORAI3 relative resistance to pH require further investigations. Beck et al. showed that ectopically expressed ORAI3 is more significantly affected by low and high pH by comparison to our results[46]. The reasons for this apparent discrepancy are unknown but the presence of native ORAI isoforms during the recordings of Beck et al. could contribute to the pH sensitivity of ectopically expressed ORAI isoforms.

The pyrazole derivatives BTP2 and GSK-7975A have been extensively used as pharmacological blockers of CRAC channels. Derler et al. reported that GSK-7975A inhibited CRAC currents mediated by ectopically expressed ORAI1 and ORAI3 in wildtype HEK293 cells [37]; the effects of GSK-7975A on CRAC currents mediated by ORAI2 was not tested. We show that 10 μ M of either BTP2 (Fig. 1) or GSK-7975A (Fig. 2) significantly inhibits ORAI1 and ORAI2, while causing only a partial inhibition of ORAI3. Interestingly, the Synta66 compound, which gained significant attention recently as a CRAC channel blocker[34, 37, 39], caused a significant inhibition of ORAI1 while potentiating ORAI2 and having no effect on ORAI3 when used at 10 μ M (Fig. 3).

Recently, we discovered that the compound IA65 (3-bromo-1,1,1-trifluoro-3-phenylpropan-2-one), is a native CRAC channel enhancer in various cell types, including MCF7 breast cancer cells, primary smooth muscle cells and skeletal muscle fibers [26]. We used CRISPR/Cas9 in MCF7 cells in which SOCE is mediated by a mixture of ORAI1 and ORAI3 to demonstrate that IA65 is an ORAI1 enhancer, having only marginal potentiating

effects on ORAI3. Here, we tested side by side the effects of IA65 (10 μ M) on all three ORAI isoforms. We confirmed our previous findings that IA65 significantly potentiated ORAI1 while only mildly potentiating ORAI3-mediated I_{CRAC} . Surprisingly, IA65 slightly inhibited I_{CRAC} mediated by ORAI2 (Fig. 4).

Because Synta66 and IA65 had both inhibitory and potentiating effects on I_{CRAC} mediated by different ORAI isoforms, we chose these two compounds to perform further studies testing their effects on CRAC currents mediated by ORAI concatenated dimers, namely ORAI1–1, ORAI1–2, ORAI1–3 and ORAI2–3. Synta66 significantly inhibited I_{CRAC} mediated by ORAI1–1 concatenated homodimers to a similar extent to that of ORAI1. However, Synta66 only partially inhibited I_{CRAC} mediated by ORAI1–2 and ORAI1–3 concatenated heterodimers, while having no effect on ORAI2–3 (Fig. 5), suggesting that within an ORAI heteromer the Synta66 pharmacological profiles of ORAI1 and ORAI3 dominate over that of ORAI2. IA65 significantly potentiated I_{CRAC} mediated by ORAI1–1 concatenated homodimers to a similar extent to that of ORAI1. However, IA65 slightly inhibited I_{CRAC} mediated by ORAI1–2 while slightly potentiating ORAI1–3 and ORAI2–3 concatenated heterodimers (Fig. 6), suggesting that within an ORAI heteromer IA65 pharmacological profile of ORAI2 dominates over that of ORAI1. The stoichiometry of native CRAC channels is likely more complex, involving variable numbers of ORAI isoform subunits within each hexamer. Assuming the position of each ORAI isoform within a hexameric CRAC channel is biologically relevant, native CRAC channels count 92 different combinations[22]. Nevertheless, Synta66 and IA65 could prove useful in understanding the molecular composition of SOCE in neuronal cell types where ORAI2 appears to be the dominant ORAI[30]. Since STIM proteins associate closely with ORAI and become an integral part of the CRAC channel during its activation, the potential role of the compounds studied herein in altering STIM/ORAI interactions and/or their ability to differentially affect STIM1 versus STIM2 requires future FRET and patch clamp investigations.

In summary, our findings define unique pharmacological features of each ORAI isoform and how heteromeric ORAI assemblies alter this pharmacology. Use of these differentially selective pharmacological activators and inhibitors of CRAC could help determine the molecular composition and the relative contribution of each ORAI isoform to native CRAC channels in various cell types and primary tissues.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- ORAI channel isoforms are distinctly affected by pH and various pharmacological inhibitors and activators
- Synta66 inhibits ORAI1 and potentiates ORAI2 whereas IA65 potentiates ORAI1 and inhibits ORAI2
- ORAI heteromerization through concatenated constructs shapes the pharmacological profiles of CRAC channels
- Distinct ORAI isoform pharmacology could be useful for determining the relative contribution of each ORAI isoform to native CRAC channels in various primary cells.

10 μ M BTP2

Figure 1. Differential effects of BTP2 on ORAI isoforms.

(A-C) Representative time course of I_{CRAC} mediated by either ORAI1, ORAI2 or ORAI3 (at -100 mV) after whole-cell mode with a pipette solution containing 20 mM BAPTA. 10 μ M BTP2 was applied to the bath when current reached steady state. (D-F) Representative I/V relationships were taken from (A-C) where indicated by the color-coded asterisks. (G-I) Data from several recordings representing steady state I_{CRAC} current density (at -100 mV) before and after addition of BTP2 represented as boxplots with the 25th to 75th percentile range, mean and median for each condition. Data were statistically analyzed using Pair-Sample t-Test (** $p < 0.01$ and *** $p < 0.001$).

10 μ M GSK-7975A

Figure 2. Differential effects of GSK-7975A on ORAI isoforms.

(A-C) Representative time course of I_{CRAC} mediated by either ORAI1, ORAI2 or ORAI3 (at -100 mV) after whole-cell mode with a pipette solution containing 20 mM BAPTA. 10 μ M GSK-7975A was applied to the bath when current reached steady state. (D-F) Representative I/V relationships were taken from (A-C) where indicated by the color-coded asterisks. (G-I) Data from several recordings representing steady state I_{CRAC} current density (at -100 mV) before and after addition of GSK-7975A represented as boxplots with the 25th to 75th percentile range, mean and median for each condition. Data were statistically analyzed using Pair-Sample t-Test (** $p < 0.001$).

10 μ M Synta66

Figure 3. Differential effects of Synta66 on ORAI isoforms.

(A-C) Representative time course of I_{CRAC} mediated by either ORAI1, ORAI2 or ORAI3 (at -100 mV) after whole-cell mode with a pipette solution containing 20 mM BAPTA. 10 μ M Synta66 was applied to the bath when current reached steady state. At the end of recording, 5 μ M Gd^{3+} was subsequently applied to the bath. (D-F) Representative I/V relationships were taken from (A-C) where indicated by the color-coded asterisks. (G-I) Data from several recordings representing steady state I_{CRAC} current density (at -100 mV) before and after addition of Synta66 represented as boxplots with the 25th to 75th percentile range, mean and median for each condition. Data were statistically analyzed using Pair-Sample t-Test (** $p < 0.01$ and ns, not significant).

10 μ M IA65

Figure 4. Differential effects of IA65 on ORAI isoforms.

(A-C) Representative time course of I_{CRAC} mediated by either ORAI1, ORAI2 or ORAI3 (at -100 mV) after whole-cell mode with a pipette solution containing 20 mM BAPTA. 10 μ M IA65 was applied to the bath when current reached steady state. At the end of recording, 5 μ M Gd^{3+} was subsequently applied to the bath. (D-F) Representative I/V relationships were taken from (A-C) where indicated by the color-coded asterisks. (G-I) Data from several recordings representing steady state I_{CRAC} current density (at -100 mV) before and after addition of IA65 represented as boxplots with the 25th to 75th percentile range, mean and median for each condition. Data were statistically analyzed using Pair-Sample t-Test (* $p < 0.05$ and ** $p < 0.01$).

10 μ M Synta66

Figure 5. Effects of Synta66 on ORAI concatenated dimers.

(A-D) Representative time course of I_{CRAC} mediated by either ORAI1-1, ORAI1-2, ORAI1-3 or ORAI2-3 concatenated dimers (at -100 mV) after whole-cell mode with a pipette solution containing 20 mM BAPTA. 10 μ M Synta66 was applied to the bath when current reached steady state. At the end of recording, 5 μ M Gd^{3+} was subsequently applied to the bath. (E-H) Representative I/V relationships were taken from (A-D) where indicated by the color-coded asterisks. (I-L) Data from several recordings representing steady state I_{CRAC} current density (at -100 mV) before and after addition of Synta66 represented as boxplots with the 25th to 75th percentile range, mean and median for each condition. Data were statistically analyzed using Pair-Sample t-Test (*p<0.05, **p<0.01, ns not significant).

10 μ M IA65

Figure 6. Effects of IA65 on ORAI concatenated dimers.

(A-D) Representative time course of I_{CRAC} mediated by either ORAI1-1, ORAI1-2, ORAI1-3 or ORAI2-3 concatenated dimers (at -100 mV) after whole-cell mode with a pipette solution containing 20 mM BAPTA. 10 μ M IA65 was applied to the bath when current reached steady state. At the end of recording, 5 μ M Gd^{3+} was subsequently applied to the bath. (E-H) Representative I/V relationships were taken from (A-D) where indicated by the color-coded asterisks. (I-L) Data from several recordings representing steady state I_{CRAC} current density (at -100 mV) before and after addition of IA65 represented as boxplots with the 25th to 75th percentile range, mean and median for each condition. Data were statistically analyzed using Pair-Sample t-Test (*p < 0.05, **p < 0.01).