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Deletion of Orai2 augments endogenous CRAC currents and degranulation in mast cells leading to enhanced anaphylaxis

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ABSTRACT

All three members of the Orai family of cation channels–Orai1, Orai2 and Orai3–are integral membrane proteins that can form store-operated Ca^{2+} channels resembling endogenous calcium release-activated channels (CRAC) in many aspects. Loss of function studies in human and murine models revealed many functions of Orai1 proteins not only for Ca^{2+} homeostasis, but also for cellular and systemic functions in many cell types. By contrast, the knowledge regarding the contribution of Orai2 and Orai3 proteins in these processes is sparse. In this study, we report the generation of mouse models with targeted inactivation of the Orai2 gene to study Orai2 function in peritoneal mast cells (PMC), a classical cell model for CRAC channels and Ca^{2+}-dependent exocytosis of inflammatory mediators. We show that the Ca^{2+} rise triggered by agonists acting on high-affinity Fc receptors for IgE or on MAS-related G protein-coupled receptors is significantly increased in Orai2-deficient mast cells. Ca^{2+} entry triggered by depletion of intracellular stores (SOCE) is also increased in Orai2$^{-/-}$ PMCs at high (2 mM) extracellular Ca^{2+} concentration, whereas SOCE is largely reduced upon re-addtion of lower (0.1 mM) Ca^{2+} concentration. Likewise, the density of CRAC currents, Ca^{2+}-dependent mast cell degranulation, and mast cell-mediated anaphylaxis are intensified in Orai2-deficient mice. These results show that the presence of Orai2 proteins limits receptor-evoked Ca^{2+} transients, store-operated Ca^{2+} entry (SOCE) as well as degranulation of murine peritoneal mast cells but also raise the idea that Orai2 proteins contribute to Ca^{2+} entry in connective tissue type mast cells in discrete operation modes depending on the availability of calcium ions in the extracellular space.

1. Introduction

All three members of the Orai family of cation channels– consisting of Orai1, Orai2, and Orai3– are sufficient to build store-operated calcium entry (SOCE) channels when heterogously expressed in cells together with STIM1 and/or STIM2 proteins [1–4]. The SOCE pathway was described over 30 years ago [5], which was followed, several years afterwards, by the decription of calcium release-activated calcium (CRAC) currents mediating SOCE in mast cells [6], and Jurkat T-cells [7]. SOCE, as well as Ca^{2+} entry triggered by various (patho)physiological stimuli, is essential for numerous cellular responses in both cell types [8–10]. Numerous studies applying loss of function mutations or pore mutations in the Orai1 protein supported the concept of Orai1 as a key constituent of CRAC channels [11,12], as well as of other types of SOCE-mediating channels in various cell types [13,14]. By contrast, to date, very few studies adressed the relevance of Orai2 proteins for SOCE and/or associated cell functions [15]. For instance, in Orai1-deficient mice [16], SOCE is substantially, but not completely reduced in several types of immune cells, including mast cells. In another mouse model where the Orai1 gene was targeted by a gene trap approach [17], CRAC currents (I_{CRAC}) are reduced by 66% in bone marrow-derived mast cells, and Ca^{2+} entry evoked by stimulation of high-affinity Fc receptors for IgE (FcεRI) is reduced to a similar extent, accompanied by a reduction of the release of inflammatory mediators. By comparison, current knowledge about the functional role of Orai2 proteins is mostly based on the studies that applied heterologous expression of the Orai2 cDNA, which leads to a pronounced enhancement of both SOCE and I_{CRAC} with its typical current-voltage relationship when co-expressed with STIM1, similarly to the results previously obtained by Orai1 expression [17–21]. While the ion selectivity of the three Orai isoforms is very similar, current densities evoked by Orai2 expression are in most cases lower as compared to Orai1 expression. Gross et al. [22], reported two

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Orai2 splice variants in the mouse: Orai2L and Orai2S. When either splice variant was expressed together with STIM1, the increase in I_{CRAC} was much lower in RBL-2H3 cells than in HEK 293 cells, suggesting a background-dependent action [22]. Expression of either Orai2 splice variant without STIM1 in RBL-2H3 cells leads to a reduction in the endogenous I_{CRAC}. This suggests that both Orai2 variants may play a dominant negative role in the formation of CRAC channels, possibly by conferring a higher sensitivity to inactivation by internal Ca^{2+} ions to the channel [22], which might be brought about by the structural differences in the C-terminus of the individual Orai proteins [15,20], and by the abundance of STIM1 proteins [23]. On the other hand, it is known that also overexpression of Orai1 results in decreased CRAC channel activity in RBL cells reflecting a requirement of certain coupling stoichiometry between STIM1 and Orai1 [24].

Downregulation of Orai2 changed SOCE neither in HEK cells [25], nor in pulmonary artery smooth muscle cells [26]. Recently, Inamaya et al. [27] showed that downregulation of Orai2 in OUMS-27 cells, a cell line derived from human chondrocytes, resulted in an increase in SOCE. Similarly, transfection with the Orai2 encoding cDNA of OUMS-27 cells results in a decrease in SOCE. Using several independent experimental approaches, this study demonstrates heteromer formation of Orai1 and Orai2 in OUMS-27 cells, thus further supporting the possibility that Orai2–together with Orai1 and STIM1 proteins–is a part of the CRAC channel complex, possibly acting as an inhibitory regulator [27]. However, although the Orai2 gene was discovered over 10 years ago [28–30], reports on cells with a complete inactivation of the Orai2 gene and its consequences on SOCE and/or I_{CRAC} were still lacking until recently. It has been speculated that a complete inactivation of the Orai2 gene in mice might be hampered by the fact that mice exhibit an intronless Orai2 gene on chromosome 16, in addition to the Orai2 gene locus on chromosome 5, that gives rise to Orai2L and Orai2S [22]. Very recently, it was reported that deletion of Orai2 increases SOCE in mouse T cells, which was explained by the ability of ORAI2 to form heteromeric channels with ORAI1 and to attenuate CRAC channel function [31]. The consequences of Orai2 deletion in primary mouse mast cells, which represent a second classical cell model in the field of CRAC channel research and SOCE, was not investigated so far.

In this study, we examined the contribution of Orai2 proteins to endogenous I_{CRAC}, agonist-evoked as well as store-operated Ca^{2+} elevation as a key signaling event for activation of mast cells. We report the generation of mice harboring an Orai2 null ($Orai2^{-}$) or conditional ($Orai2^{fx}$) allele using gene targeting in embryonic stem cells. Furthermore, our approach yielded mice with an Orai2 L2F2 allele that functions as a YFP reporter of Orai2 expression. In peritoneal mast cells (PMCs) obtained from $Orai2^{-/-}$ mice, the expression of Orai2 transcripts is abolished, but the expression of Orai1 and Orai3 genes, as well as of other central regulators of SOCE, remains unchanged in $Orai2^{-/-}$ PMCs. Our functional analysis shows an increase in I_{CRAC}, as well as agonist-evoked Ca^{2+} elevations in $Orai2^{-/-}$ PMCs. This is accompanied by enhanced degranulation and FcεRI-mediated anaphylaxis in vivo, thereby supporting the concept of Orai2 as a negative regulator of the CRAC channel complex in PMCs.

2. Methods

2.1. Gene targeting

Cloning of the targeting vector and gene targeting was performed at ingenious targeting laboratory, Inc (USA). C57Bl/6 embryonic stem cells were transfected with 10 µg of the linearized targeting vector by electroporation. After selection with G418 antibiotic, surviving clones were expanded for PCR analysis to identify recombinant ES clones. The primers for the PCR-based screening were the following: A1: 5′-AGC ACG CAT CTC GGT CAG TAG AG–3′; SQ1: 5′-TCC CTG ACA GGA AGA GTC AGT G–3′; LAN1: 5′-CCA GAG GCC ACT TGT GTA GC–3′; LOX1: 5′-TTG CAG TTG CCC CGG ATT GAG–3′; SC1: 5′-CAT CTA CCT GCC CCT ATC CAG ATG–3′; SCR2: 5′-ACC TTG GGA CCA CCT CAT CAG AAG–3′. The screening primer A1 was designed upstream of the short homology arm (SA) outside the 5′ region. PCR reactions using A1 with the SCR2 primer (located within the Neo cassette) amplify a 2.63 kb fragment. Clones 113, 183, 213, 314, and 353 were identified as positive and selected for expansion. All five clones were expanded and reconfirmed for SA integration by PCR using primers A1 and SCR2, which yields a 2.63 kb fragment (not shown). Sequencing was performed on purified PCR DNA to confirm the presence of the junction of genomic DNA and the YFP/NEO cassette using the SQ1 primer. The presence of the distal loxP site was confirmed by a PCR using LOX1 and SC1 primers, resulting in a 448 bp fragment (the size of the wild type product is 382 bp). Confirmation of the retention of the distal LoxP site was performed by PCR using the SC1 and LAN1 primers. This reaction produces a 4.69 kb fragment. Sequencing was performed on purified PCR DNA to confirm the presence of the distal LoxP cassette using the LOX1 primer.

Secondary confirmation of positive clones identified by PCR was performed by Southern Blot analysis. ES cell DNA was digested with Ssp I, and electrophoretically separated on a 0.8% agarose gel. After transfer to a nylon membrane, the digested DNA was hybridized with a probe targeted against the 3′ external region. DNA from C57Bl/6 (B6) mouse strain was used as a wild type control. The expected sizes are indicated in Suppl. Fig. 1. The 3‘ Probe (476 bp) was amplified using the following primers: PB 3: 5′-ACT GGC TGA TGT CGC CAG AAC–3′; PB 4: 5′-AGA AGC TGA GAT GGT GCG TCT G-3′. Positive clones were further confirmed by Southern Blotting analysis using a 5′ internal probe (EcoRV digestion). The 5′ Probe (448 bps) was amplified using following primers: PB 1: 5′- AGT GGA GCT GTG AGC CAA GTG-3′; PB 2: 5′-TGG TCT TGC CTA GTA TGT CCA TGG −3′.

Positive clones were further analyzed by Southern Blotting analysis using a Neo probe. DNA was digested with Ssp I, and the digested DNA was hybridized with a probe targeted against the Neo cassette. The Neo Probe (436 bps) was amplified using the following primers: NeoF: 5′-ACA AGA TGG ATT GCA CGC AGG TTC-3′; NeoR: 5′-ATG GAT ACT TTC TCG GCA GGA GCA-3′. Clones 113, 183, 213, 314, and 353 were confirmed as correctly targeted. The karyotype analysis was performed by G-banding. For each clone, 20 metaphase spreads were analyzed using the Applied Spectral Imaging's BandView software and the euploidy percentage of each culture was calculated following the Cold Spring Harbor Laboratory's chromosome counting protocol. Clone 183 which had 81% of euploidy was used for blastocyst injection. To this end, ES cells were propagated in GS2M Basal Medium (StemCell Inc., UK) and injected into blastocyst of the Balb/c strain at the IBF (Interfakultäre Biomedizinische Forschungseinrichtung) Animal facility of Heidelberg University. Founder animals were crossed to C57Bl6/N females.

Heterozygous $Orai2^{+/L2F2}$ mice were crossed with Flp deleter mice [32], to obtain $Orai2^{+/fx}$ mice. Deletion of the YFP/NEO cassette was confirmed by PCR using the primers: VT-1: 5′-TCC CTG ACA GGA AGA GTC AGT G-3′and VT2: 5′-AAT GAAGAG CTG GGG CAT GG-3′. $Orai2^{+/fx}$ mice were crossed with Cre deleter mice [33] to obtain $Orai2^{+/-}$ mice which was confirmed by PCR using the primers: VT1 and VT3: 5′-CAT CTA CCT GCC CCT ATC CAG ATG-3′. Alternatively, $Orai2^{+/fx}$ mice were crossed with Mcpt5-Cre mice [34] to obtain $Orai2^{fx/fx}$; Mcpt-5 Cre^{+} mice in the second generation. Inheritence of the Cre transgene was confirmed by PCR using primers Mcpt5-CreFor: 5′-ACA GTG GTA TTC CCG GGG AGT GT-3′ and Mcpt5-CreRev: 5′-GTC AGT GCG TTC AAA GGC CA-3′.

2.2. Peritoneal mast cell (PMC) culture

For the isolation of PMCs, peritoneal cells were gained by washing the peritoneal cavity (peritoneal lavage) of 2–3 mice with RPMI medium. Cells were centrifuged and resuspended in RPMI Medium containing 20% FCS and 1% PenStrep. The cells were further cultured