Biochimica et Biophysica Acta 1823 (2012) 1242–1251

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Capacitative and non-capacitative signaling complexes in human platelets

Alejandro Berna-Erro, Carmen Galan, Natalia Dionisio, Luis J. Gomez, Gines M. Salido, Juan A. Rosado *

Department of Physiology, Cell Physiology Research Group, University of Extremadura, Cáceres, Spain

ARTICLE INFO

Article history:
Received 26 January 2012
Received in revised form 18 May 2012
Accepted 21 May 2012
Available online 26 May 2012

Keywords:
Orai1
Orai2
Orai3
STIM1
STIM2
TRPC

ABSTRACT

Discharge of the intracellular Ca^{2+} stores activates Ca^{2+} entry through store-operated channels (SOCs). Since the recent identification of STIM1 and STIM2, as well as the Orai1 homologs, Orai2 and Orai3, the protein complexes involved in Ca^{2+} signaling needs re-evaluation in native cells. Using real time PCR combined with Western blotting we have found the expression of the three Orai isoforms, STIM1, STIM2 and different TRPCs in human platelets. Depletion of the intracellular Ca^{2+} stores with thapsigargin, independently of changes in cytosolic Ca^{2+} concentration, enhanced the formation of a signaling complex involving STIM1, STIM2, Orai1, Orai2 and TRPC1. Furthermore, platelet treatment with the dyacylglicerol analog 1-oleoyl-2-acetyl-sn-glycerol (OAG) resulted in specific association of Orai3 with TRPC3. Treatment of platelets with arachidonic acid enhanced the association between Orai1 and Orai3 in human platelets and overexpression of Orai1 and Orai3 in HEK293 cells increased arachidonic acid-induced Ca^{2+} entry. These results indicate that Ca^{2+} store depletion results in the formation of exclusive signaling complexes involving STIM proteins, as well as Orai1, Orai2 and TRPC1, but not Orai3, which seems to be involved in non-capacitative Ca^{2+} influx in human platelets.

1. Introduction

Receptor-operated Ca^{2+} entry is a ubiquitous and important mechanism for Ca^{2+} influx in excitable and non-excitable cells. This mechanism comprises Ca^{2+} entry pathways activated by receptor occupation, from the non-capacitative Ca^{2+} currents via receptor-channels themselves or through channels operated by second messengers to the capacitative Ca^{2+} entry pathway activated by discharge of the intracellular Ca^{2+} pools via store-operated Ca^{2+} channels (SOCs) [1]. Capacitative or store-operated Ca^{2+} entry (SOCE) is a complex mechanism controlled by the filling state of the intracellular Ca^{2+} stores. In 2005, the protein STIM1 was identified as the endoplasmic reticulum (ER) Ca^{2+} sensor that communicates the information of the filling state of the ER to SOCs located in the plasma membrane [2]. Concerning SOCs, the canonical human homologs of *Drosophila* transient receptor potential channels (TRPC), particularly TRPC1, have been presented as candidates to mediate non Ca^{2+}-selective capacitative currents (I_{SOC}) [3]. In 2006, Orai1 was presented as the pore unit of the channel that mediate I_{CRAC}, the best characterized and Ca^{2+}-selective capacitative current [4,5], and a number of studies demonstrated that co-expression of STIM1 and Orai1 is able to enhance I_{CRAC} [6] or to successfully reconstitute I_{CRAC}-like signals in HEK-293 cells [7]. Isoforms of these proteins, STIM2, Orai2 and Orai3, have also been identified, although their role in intracellular Ca^{2+} homeostasis is less characterized [8].

STIM2 is almost ubiquitously expressed in human tissues and has been found located in the ER membrane and in the membrane of acidic intracellular Ca^{2+} stores [9–11], but not in the plasma membrane in contrast to STIM1 [10,12]. Nevertheless, the function of STIM2 is still unclear. By using STIM2 knockdown expression, it has been suggested that STIM2 acts as a regulator that stabilizes resting Ca^{2+} concentration in the cytosol and the ER [13]. Concerning SOCE, STIM2 has been shown to be able to interact with Orai proteins and TRPC1 channels [6,14], thus suggesting a potential role in SOCE. Studies performed by Gill's group suggested that STIM2 inhibits SOCE when expressed alone [10], but induces constitutive SOCE when co-expressed with Orai1 [6]. Further studies have reported different degrees of attenuation of SOCE in the absence of STIM2 expression in a variety of cells [15,16], and STIM2-mediated Ca^{2+} entry via two store-dependent and store-independent modes in HEK293 cells [14].

Orai2 and Orai3 are also widely expressed in different human tissues [17,18]. Knockdown gene expression in a variety of cell lines has revealed that Orai2 and Orai3 have a minor role in SOCE than Orai1 [19–21]. The functional properties of Orai3 have been shown to be slightly different to those of Orai1 and Orai2. The latter show similarly fast activation kinetics while Orai3 shows slower activation. In addition, Orai3 is more permeant to monovalent cations than Orai1 and Orai2 [22]. Co-expression of STIM1 and Orai1 or Orai2 channels increased SOCE in HEK293 cells [7] and mouse [23], indicating that these isoforms interact and are activated by STIM1. In contrast, no significant increases in SOCE were found in cells co-expressing STIM1 and Orai3 [7].

In the present study we have investigated the expression and interaction of the different isoforms of Orai and STIM proteins and the

* Corresponding author at: Department of Physiology, University of Extremadura, Caceres, 10071, Spain. Tel.: +34 927257139; fax: +34 927257110.
E-mail address: jarosado@unex.es (J.A. Rosado).

0167-4889/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamcr.2012.05.023

most relevant TRPC subfamily members in human platelets in response to store depletion or stimulation with the soluble analog of the second messenger diacylglycerol, 1-oleoyl-2-acetyl-sn-glycerol (OAG). Our results suggest that, while Orai1 and Orai2 operate in a STIM-dependent manner, Orai3 does not interact with STIM isoforms upon store depletion in these cells.

2. Materials and methods

2.1. Materials

Apyrase (grade VII), aspirin, thapsigargin (TG), bovine serum albumin (BSA), phenylmethylsulfonyl fluoride (PMSF), arachidonic acid, 1-oleoyl-2-acetyl-sn-glycerol (OAG) and rabbit anti-Orai1 antibody were purchased from Sigma (Madrid, Spain). Goat anti-hTRPC6 polyclonal antibody, horseradish peroxidase-conjugated goat anti-rabbit IgG antibody and horseradish peroxidase-conjugated donkey anti-goat IgG antibody were from Santa Cruz (Santa Cruz, CA, U.S.A). Rabbit anti-TRPC1 antibody was from Alomone Labs (Jerusalem, Israel). Rabbit anti-TRPC3 polyclonal antibody, rabbit anti Orai-2 polyclonal antibody and rabbit anti Orai-3 polyclonal antibody were from Abcam (Cambridge, U.K.). Mouse anti STIM1 antibody was from BD Transduction Laboratories (Frankin Lakes, NJ, U.S.A). Rabbit polyclonal anti-STIM2 antibody was from Cell Signaling (Boston, MA, U.S.A). Hyperfilm ECL was from Amersham (Buckinghamshire, U.K.). Transpass transfection reagent was from Izasa (Madrid, Spain). Protein A-agarose was from Upstate Biotechnology Inc. (Madrid, Spain). Enhanced chemiluminescence detection reagents and rabbit anti-mouse IgG were from Pierce (Cheshire, U.K.). All other reagents were of analytical grade.

2.2. Platelet preparation

Platelets were prepared as previously described [24] as approved by Local Ethical Committees and in accordance with the Declaration of Helsinki. Briefly, blood was obtained from healthy drug-free volunteers and mixed with one-sixth volume of acid/citrate dextrose anticoagulant containing (in mM): 85 sodium citrate, 78 citric acid and 111 D-glucose. Platelet-rich plasma (PRP) was then prepared by centrifugation for 5 min at 700 ×*g* and aspirin (100 μM) and apyrase (40 μg/mL) were added. For dimethyl BAPTA loading, the platelet-rich plasma was incubated at 37 °C with 10 μM dimethyl BAPTA acetoxymethyl ester for 20 min. Cells were then collected by centrifugation at 350 ×*g* for 20 min and resuspended in HEPES-buffered saline (HBS), pH 7.45, containing (in mM): 145 NaCl, 10 HEPES, 10 D-glucose, 5 KCl, 1 MgSO4 and supplemented with 0.1% BSA and 40 μg/mL apyrase.

2.3. Cell culture

Human embryonic kidney 293 (HEK293) cells were obtained from the American Type Culture Collection (Barcelona, Spain) and cultured in Dulbecco's modified Eagle's medium, supplemented with 10% heat-inactivated fetal bovine serum, in a 37 °C incubator with 5% CO_2. At the time of the experiments cells were suspended in HEPES-buffered saline (HBS) containing (in mM): 145 NaCl, 10 HEPES, 10 D-glucose, 5 KCl, 1 MgSO4, 1 mM CaCl2, pH 7.45. Orai1 and Orai3 cDNA plasmids were kindly provided by Dr. Trebak and Dr. Romanin, respectively. Cells were transiently transfected with plasmids using the transpass transfection reagent, and used 1–3 d after transfection as previously described [25].

2.4. Real time-PCR

RNA was extracted from isolated human platelets using TRIzol® reagent (Invitrogen, Carlsbad, CA) according to manufacturer's specifications, and 2.5 μg of total RNA was subsequently reverse-transcribed to single-strand cDNA using SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen, Carlsbad, CA). Single-strand cDNA products were directly used for PCR amplification performed with an Eppendorff Mastercycler® thermal cycler (Eppendorf AG, Hamburg-Eppendorf, Germany). PCR reagents such as Taq polymerase and buffers were purchased from TAKARA (Takara Bio Inc., Otsu, Shiga, Japan). PCR products were obtained using the following cycling conditions: 96 °C for 2 min, followed by 35 cycles of 96 °C for 15 s, 48–56 °C for 25 s, 72 °C for 30 s, and finished with 72 °C for 10 min. Primers listed in Table 1 were used to amplify cDNA transcripts of human *TRPC1*, *TRPC3*, *TRPC6*, *Orai1*, *Orai2*, *Orai3*, *STIM1* and *STIM2* genes. PCR products were separated by electrophoresis in 1.5% agarose gels (Roth, Germany) and the resulting bands were documented and subsequently isolated from gels using UltraClean® PCR Clean-Up Kit (MO BIO Laboratories Inc., Carlsbad, CA). Amplification of the desired genes was confirmed by sequenciation of PCR products (STAB-SAIUex, University of Extremadura, Spain).

Table 1
Primers.

Gene	Forward (5′–3′)	Reverse (5′–3′)	Bp.	Anneal T. (°C)	Ref.
hTRPC1	tgcgtagatgtgcttgggag	atgctctcagaattggatcc	377	54.6	[51]
hTRPC3	cgatctggtatgagaacc	cagtccatgtaaactggg	216	48	
hTRPC6	tcatcatggtgtttgtggc	gcaaaacaatgaccattgtaa	237	56.5	[52]
hOrai1	agcaacgtgcacaatctcaa	gtcttatggctaaccagtga	344	56.5	[23]
hOrai2	cggccataagggcatggatt	ttgtggatgttgctcacggc	333	56.5	[23]
hOrai3	ctcttccttgctgaagttgt	cgattcagttcctctagttc	354	56.5	[23]
hSTIM1	cagtgaaacacagcaccttcc	aagagcactgtatccagagcc	213	56.5	[53]
hSTIM2	ccagggctttcactgtgatt	cctcggcttaaggttgtgaa	147	60	[54]
hβ-actin	agcgagcatcccccaaagtt	gggcacgaaggctcatcatt	285	48–60	[55]

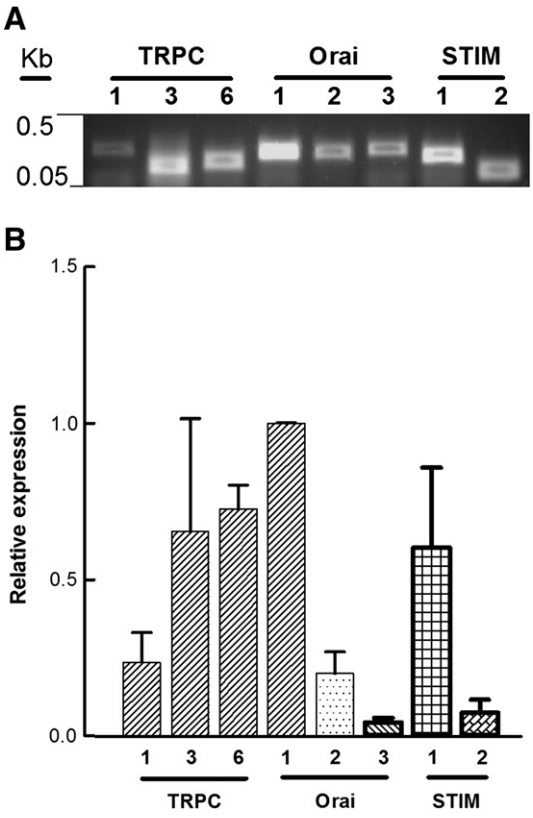

Fig. 1. Quantification of Orai, STIM and TRPC mRNA transcripts in human platelets. A, Representative picture of semi-quantitative RT-PCR products separated by electrophoresis in agarose gels. B, qRT-PCR expression analysis of TRPC(1, 3, 6), Orai(1–3) and STIM(1–2) mRNA transcripts in human platelets. Values were normalized to β-actin expression and represented as (mean expression relative to Orai1 ± S.E.M; $n = 3$).