Cell Calcium 74 (2018) 14–28

Contents lists available at ScienceDirect

Cell Calcium

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

Evidence for the interaction of peroxiredoxin-4 with the store-operated calcium channel activator STIM1 in liver cells

Ka Cheung Tam, Eunus Ali, Jin Hua, Tim Chataway, Greg J. Barritt*

Discipline of Medical Biochemistry, College of Medicine and Public Health, Flinders University, Adelaide, South Australia, 5001, Australia

ARTICLE INFO

Keywords:
Store-operated Ca^{2+} channels
STIM1
Orai1
Liver
Subcellular fractionation
peroxiredoxin-4

ABSTRACT

Ca^{2+} entry through store-operated Ca^{2+} channels (SOCs) in the plasma membrane (PM) of hepatocytes plays a central role in the hormonal regulation of liver metabolism. SOCs are composed of Orai1, the channel pore protein, and STIM1, the activator protein, and are regulated by hormones and reactive oxygen species (ROS). In addition to Orai1, STIM1 also interacts with several other intracellular proteins. Most previous studies of the cellular functions of Orai1 and STIM1 have employed immortalised cells in culture expressing ectopic proteins tagged with a fluorescent polypeptide such as GFP. Little is known about the intracellular distributions of endogenous Orai1 and STIM1. The aims are to determine the intracellular distribution of endogenous Orai1 and STIM1 in hepatocytes and to identify novel STIM1 binding proteins. Subcellular fractions of rat liver were prepared by homogenisation and differential centrifugation. Orai1 and STIM1 were identified and quantified by western blot. Orai1 was found in the PM (0.03%), heavy (44%) and light (27%) microsomal fractions, and STIM1 in the PM (0.09%), and heavy (85%) and light (13%) microsomal fractions. Immunoprecipitation of STIM1 followed by LC/MS or western blot identified peroxiredoxin-4 (Prx-4) as a potential STIM1 binding protein. Prx-4 was found principally in the heavy microsomal fraction. Knockdown of Prx-4 using siRNA, or inhibition of Prx-4 using conoidin A, did not affect Ca^{2+} entry through SOCs but rendered SOCs susceptible to inhibition by H_2O_2. It is concluded that, in hepatocytes, a considerable proportion of endogenous Orai1 and STIM1 is located in the rough ER. In the rough ER, STIM1 interacts with Prx-4, and this interaction may contribute to the regulation by ROS of STIM1 and SOCs.

1. Introduction

Store-operated Ca^{2+} channels (SOCs) in the plasma membrane (PM), and Ca^{2+} entry through these channels (SOCE), play essential roles in the regulation by intracellular Ca^{2+} of cell growth, death and function in almost all animal cell types [1]. SOCE replenishes the ER Ca^{2+} stores during and after $InsP_3$-mediated Ca^{2+} release induced by hormones and other extracellular signals. In some situations SOCE contributes directly to changes in the cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) induced by extracellular signals [1,2]. In addition to hormonal signals, reactive oxygen species (ROS) can also alter SOCE in both normal and pathological conditions (reviewed in [3,4]). Most SOCs are composed of Orai1 polypeptides, which constitute the channel pore located in the PM, and STIM1 polypeptides located in the endoplasmic reticulum (ER), which activate Orai1 (reviewed in [1]).

SOC activation is initiated by a decrease in the Ca^{2+} concentration in the lumen of a sub-region of the ER which is devoid of ribosomes and located in ER-PM junctions at the periphery of the cell [1,5,6]. This leads to the dissociation of Ca^{2+} from the luminal E, F hand-sterile alpha (EF-SAM) Ca^{2+} binding motif in the N-terminal of STIM1, conformational changes in STIM1, movement of STIM1 in the plane of the ER membrane, interaction of the cytoplasmic CRAC activation domain (CAD) in the C-terminal of STIM1 with Orai1 located in the PM, and formation of an active functional SOC [1,7]. Functional SOCs are composed of a hexamer of Orai1 polypeptides and approximately 4 STIM1 polypeptides [1,7]. It is generally thought that in order to achieve SOCE activation, Orai1 must be present in the PM and STIM1 in the peripheral ER, located in close proximity to the plasma membrane [1]. The results of other studies have provided evidence for the interaction of STIM1 with numerous other intracellular proteins (reviewed

Abbreviations: $[Ca^{2+}]_{cyt}$, cytoplasmic free Ca^{2+} concentration; Ca^{2+}_{ext}, extracellular Ca^{2+}; ER, endoplasmic reticulum; PM, plasma membrane; SERCA, sarco/endoplasmic reticulum (Ca^{2+} + Mg^{2+})ATP-ase; PMCA, plasma membrane (Ca^{2+} + Mg^{2+})ATP-ase; SOCE, store-operated Ca^{2+} entry; SPCA, secretory pathway Ca^{2+} ATP-ase; SOC, store-operated Ca^{2+} channel; STIM, stromal interaction molecule; $[Ca^{2+}]_{ext}$, extracellular free Ca^{2+} concentration; DBHQ, 2,5-di-(*tert*-butyl)-14-benzohydro-quinone; GFP, green fluorescent protein; Prx-4, peroxiredoxin-4; TRPM2, transient receptor potential melastatin 2; PMSF, phenylmethylsulfonyl fluoride; ROS, reactive oxygen species; HRP, horse radish peroxidase

* Corresponding author at: Discipline of Medical Biochemistry, College of Medicine and Public Health, Flinders University, GPO Box 2100, Adelaide, SA, 5001, Australia.
E-mail address: greg.barritt@flinders.edu.au (G.J. Barritt).

https://doi.org/10.1016/j.ceca.2018.05.002
Received 11 February 2018; Received in revised form 4 May 2018; Accepted 12 May 2018
Available online 15 May 2018
0143-4160/

in [8]). Some of these STIM1-binding proteins modulate the interaction of STIM1 with Orai1 or modulate other proteins involved in intracellular Ca^{2+} homeostasis, while others are involved in regulation of the microtubular network [8–10].

Most previous studies on the intracellular distribution of Orai1 and STIM1 have been conducted using immortalised cells in culture over-expressing ectopic Orai1 and STIM1 tagged with a fluorescence peptide such as GFP, or variants of GFP [1,11–16]. Much less is known about the distribution of endogenous Orai1 and STIM1 *in situ*. We have previously characterised SOCs in hepatocytes. These cells express functional SOCs with high selectivity for Ca^{2+}, and channel properties essentially identical to those in lymphocytes and mast cells [17]. While each of the main isoforms of STIM and Orai has been detected in rat hepatocytes and in immortalised H4IIE rat liver cells, the expression of STIM2 is lower than that of STIM1, Orai2 is barely detectable, and expression of Orai3 is much lower than that of Orai1 [18]. Previous studies, in which expression of STIM1 and Orai1 has been knocked down by siRNA, indicate that SOCs in rat hepatocytes and in H4IIE rat liver cells are principally composed of the STIM1 and Orai1 polypeptides [17,19]. Our previous studies employing GFP- or Cherry-STIM1 have shown that, upon the release of Ca^{2+} from the ER, STIM1 forms puncta and moves to the PM [20,21].

The aims of this study were to investigate the intracellular distribution of endogenous Orai1 and STIM1 in hepatocytes and to seek to identify additional novel proteins which may modify the action of STIM1. The experiments have employed the preparation and analysis of subcellular fractions of rat liver. The bulk of the liver is composed of hepatocytes which are the principle cell type responsible for liver-specific functions [22]. Moreover, liver tissue provides a good starting point for subcellular fractionation since it is easily homogenised, and high yields of each subcellular fraction can be obtained [23]. A subcellular fractionation approach, rather than an alternative one based on immunofluorescence and confocal imaging, was chosen since we and others have found that it is sometimes difficult to obtain antibodies against PM ion channel proteins such as Orai1 and members of the TRP family with sufficient specificity [24,25]. Furthermore, we wanted to employ immunoprecipitation and LC/MS to identify new STIM1 binding partners. This strategy requires relatively large amounts of protein, which can be obtained by liver homogenisation and subcellular fractionation.

2. Materials and methods

2.1. Materials

Phenylephrine was purchased from Sigma, St. Louis, MO USA; Conoidin A from the Cayman Chemical Company, whole serum (# R-1593-100) from Biosensis, Thebarton, South Australia; Protein A/G PLUS-Agarose immunoprecipitation reagent (#SC2003), normal mouse IgG (#SC-2025) and normal rabbit IgG (# sc-2027) from Santa Cruz Biotechnology, Dallas, Texas, USA, and protein ladders (markers) for western blot from BioRad Laboratories, New South Wales, Australia. The sources of the primary and secondary antibodies are listed in Table 1. The sources of all other materials were as described previously [18,26,27].

2.2. Rat liver homogenisation

Male Hooded Wistar rats weighing 250–350 g, housed in the Flinders Medical Centre Animal Facility, were provided *ad libitum* access to food and water, with a lighting cycle of 12 h. Animals received humane care, and the experimental protocols were conducted according to the criteria outlined in the "Australian Code of Practice for the Care and Use of Animals for Scientific Purposes" (National Health and Medical Research Council of Australia). Removal of the liver and homogenisation of the liver were performed as described by Lievrement et al. [28]. Rats were sedated with isoflurane then anesthetized by an intraperitoneal injection of ketamine (100 mg/kg body weight) and xylazine (8 mg/kg body weight). The homogenisation medium was composed of 250 mM sucrose, 5 mM HEPES-KOH and 1 mM EGTA, adjusted to pH 7.4, and supplemented with 1 mM dithiothreitol, and the proteolytic inhibitors 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml leupeptin, 10 μM pepstatin A, 2 μM benzamide, 5 μg/ml aprotinin, 50 μg/ml trypsin inhibitor, and 1 μg/ml *O*-phenanthroline. Homogenisation was performed using 10 passes of the loose-fitting pestle, and 3 passes of the tight-fitting pestle, of a 40 ml Dounce homogeniser (Kontes, DWK Life Sciences (Kimble)).

2.3. Preparation of subcellular fractions

The preparation of subcellular fractions was based on procedures previously developed by Lievrement et al. and Prpic et al. [28,29]. The liver homogenate was centrifuged at for 1500*g* 10 min. This yielded a low speed pellet, which contains microsomes derived from the nuclear membrane and the PM, and a supernatant, which contains mitochondria, microsomes derived from the rough and smooth ER, other organelles, and cytosolic proteins [23,28]. The pellet was resuspended in 2 ml of 250 mM sucrose, 25 mM HEPES-KOH, adjusted to pH to 7.4, containing the proteolytic inhibitors listed above (Wash Medium) to give a final volume of about 12 ml. Two different subcellular fractionation strategies, designated the "Microsomal and Plasma Membrane Fractionation" strategy and the "Mitochondria, Microsomal and Cytosolic Fractionation" strategy, were then employed. In the first (the Microsomal and Plasma Membrane Fractionation), the re-suspended low speed pellet was used to prepare a PM fraction using Percoll gradient centrifugation as described by Prpić et al. [29]. The PM fraction was finally suspended in about 1 ml of 250 mM sucrose, 50 mM Tris − HCl, pH 8. To prepare the heavy and light microsomal fractions, the low speed supernatant was centrifuged at 8000*g* for 20 min. The resulting pellet (the mitochondrial fraction) was discarded. The supernatant was centrifuged at 35,000*g* for 30 min to yield a pellet of heavy microsomes and a supernatant containing light microsomes. The 35,000*g* supernatant was then centrifuged at 100,000*g* for 60 min to yield a pellet of light microsomes and a cytosolic fraction which was discarded. The heavy and light microsomal pellets were each re-suspended by adding 6 ml Wash Medium. In the second subcellular fractionation strategy (the Mitochondria, Microsomal and Cytosolic Fractionation), the low speed pellet was retained for analysis (designated "low speed pellet") while the low speed supernatant was used to prepare a mitochondrial fraction, heavy and light microsomal fractions, and a cytosolic fraction. The low speed (1500*g*) supernatant was centrifuged at 8000*g* for 20 min. The resulting pellet (the mitochondrial fraction) was resuspended in 6 ml of Wash Medium. The supernatant was centrifuged at 35,000*g* for 30 min to yield a pellet of heavy microsomes and a supernatant, containing light microsomes. The 35,000*g* supernatant was then centrifuged at 100,000*g* for 60 min to yield a pellet of light microsomes and the cytosolic fraction. The heavy and light microsomal pellets were each resuspended by adding 6 ml Wash Medium. The concentration of protein in the liver homogenate and in each subcellular fraction was determined by the EZQ method [30], as described previously [18,27].

2.4. Treatment of livers with phenylephrine

Rats were anesthetized as described above and laparotomy performed to expose the inferior vena cava and the liver. Phenylephrine (1 mM stock solution in 0.9% (w/v) NaCl) was injected into the inferior vena cava to give an estimated initial blood concentration of 15 μM phenylephrine, based on a blood volume of 7.5 ml per 100 g body weight [31]. After 20 min, a blood sample (500 μl) was taken from the inferior vena cava, and the liver removed and homogenised as described above. During the administration of phenylephrine, the