The International Journal of Biochemistry & Cell Biology 42 (2010) 337–345

Contents lists available at ScienceDirect

The International Journal of Biochemistry & Cell Biology

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

Chemico-genetic identification of drebrin as a regulator of calcium responses

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ARTICLE INFO

Article history:
Received 14 July 2009
Received in revised form 9 November 2009
Accepted 23 November 2009
Available online 3 December 2009

Keywords:
Calcium
Actin
Cytoskeleton
Soc
NFAT

ABSTRACT

Store-operated calcium channels are plasma membrane Ca^{2+} channels that are activated by depletion of intracellular Ca^{2+} stores, resulting in an increase in intracellular Ca^{2+} concentration, which is maintained for prolonged periods in some cell types. Increases in intracellular Ca^{2+} concentration serve as signals that activate a number of cellular processes, however, little is known about the regulation of these channels. We have characterized the immuno-suppressant compound BTP, which blocks store-operated channel mediated calcium influx into cells. Using an affinity purification scheme to identify potential targets of BTP, we identified the actin reorganizing protein, drebrin, and demonstrated that loss of drebrin protein expression prevents store-operated channel mediated Ca^{2+} entry, similar to BTP treatment. BTP also blocks actin rearrangements induced by drebrin. While actin cytoskeletal reorganization has been implicated in store-operated calcium channel regulation, little is known about actin-binding proteins that are involved in this process, or how actin regulates channel function. The identification of drebrin as a mediator of this process should provide new insight into the interaction between actin rearrangement and store-operated channel mediated calcium influx.

1. Introduction

Stimulation of tyrosine kinase coupled receptors such as the T cell receptor in T-lymphocytes results in activation of phospholipase Cγ (PLCγ) which hydrolyzes phosphoinositol 4,5-bisphosphate (PIP_2) to generate the second messengers inositol 1,4,5 trisphosphate (IP_3) and diacyl glycerol (DAG) (Winslow et al., 2003). Similarly, IP_3 and DAG are generated by PLCβ downstream of G-protein coupled receptors (Hepler and Gilman, 1992). This results in increased intracellular calcium concentration $[Ca^{2+}]_i$ due to the action of IP_3 on IP_3 receptors (IP_3R) in the endoplasmic reticulum (ER) membrane which, when activated stimulate the release of ER calcium stores into the cytoplasm. Emptying of the ER calcium stores stimulates entry of extracellular calcium through store-operated channels (SOCs) or CRAC channels, thus maintaining the higher concentration of intracellular Ca^{2+}. $[Ca^{2+}]_i$ increases play a critical role in a variety of cellular processes such as transcription factor activation and gene expression, and cytoskeletal reorganization. In T cells, increased $[Ca^{2+}]_i$ results in activation of the transcription factor, nuclear factor of activated T cells (NFAT), which is essential for transcription of many cytokine genes important in generating an immune response.

Store-operated channel mediated entry of Ca^{2+} through CRAC channels is the main mechanism used by many cells to sustain an increased $[Ca^{2+}]_i$. The absence of Ca^{2+} influx through CRAC channels can severely compromise immune cell activation, proliferation, and effector functions (Feske, 2007; Gwack et al., 2007). This is underscored by the existence of one form of SCID syndrome, whose pathological roots trace to defective CRAC channel function (Gwack et al., 2007). In T-lymphocytes from the patients who are affected by this form of SCID, a missense mutation and an Arginine-to-Tryptophan amino acid (a.a.) substitution at a.a. position 91 in the first transmembrane domain of the Orai1 protein result in the ablation of all CRAC channel activity (Feske, 2007). Stromal Interaction Molecule 1 (STIM1) and Orai are integral parts of the ER-to-plasma

Abbreviations: IP_3, inositol 1,4,5 trisphosphate; DAG, diacyl glycerol; ER, endoplasmic reticulum; FLE, filopodia-like-extensions; SA, streptavidin; SOC, store-operated channel; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; BTP, 3,5-bistrifluoromethyl pyrazole; NFAT, nuclear factor of activated T cells.

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doi:10.1016/j.biocel.2009.11.019

membrane (PM) signaling system, necessary for store-operated channel entry (Hogan and Rao, 2007). STIM1 is a single-spanning membrane protein with a Ca^{2+}-binding EF-hand motif and functions as the sensor of ER luminal Ca^{2+} levels, and its reorganization in the ER allows it to transduce information directly to the plasma membrane. At the plasma membrane, STIM1 may interact with Orai to allow store-operated channel entry of Ca^{2+}. Despite experimental evidence showing that STIM1 and Orai1 are necessary and sufficient for SOCE, many questions remain about the details of the coupling mechanism between these proteins (Hewavitharana et al., 2007). A structural analysis of STIM1–Orai1 interactions by Varnai et al. (2007) implicated the presence of additional molecular components within the STIM1–Orai1 complex. Therefore, the identification of other molecules, which regulate the operation of CRAC channels, will allow us to better understand how the interactions between STIM1 and Orai1 occur and where they take place within cells. Most importantly, this will allow us to have an impact on the diseases that associate with malfunctioning states of store-operated channel Ca^{2+} entry.

Actin cytoskeletal changes have been suggested to be essential for the operation of store-operated calcium channels (Patterson et al., 1999; Hao and August, 2005b). Interestingly, actin cytoskeletal changes appear to be dispensable for ER calcium release (Patterson et al., 1999; Hao and August, 2005b). Indeed, if actin polymerization is induced with an agent such as jasplakinolide, which prevents actin depolymerization, prior to administration of a calcium ionophore, store-operated channel mediated $[Ca^{2+}]_i$ increase is completely blocked (Patterson et al., 1999). This phenomenon may best be explained by a recent model for activation of store-operated channels that has been proposed wherein the signal between the ER and the plasma membrane that activates store-operated channels involve a secretion-like mechanism which is blocked by thick cortical actin (Patterson et al., 1999). Along these lines, it was recently demonstrated that treatment of DT40 B cells with the actin depolymerizing agent latrunculin B (LatB) prior to stimulation through the B cell receptor, increased the sensitivity to B cell receptor signals. This was shown to be mediated at least in part by an increase in the intensity and duration of calcium signals (Hao and August, 2005b).

Recently, a class of compounds called BTPs (3,5-bis(trifluoromethyl)pyrazoles) was found to inhibit activation of the calcium regulated transcription factor NFAT (Trevillyan et al., 2001). Other drugs that target NFAT are currently being used clinically to prevent organ-transplant rejection by suppressing the immune system. These drugs, FK506 and cyclosporin A, work by inhibiting the phosphatase activity of the serine/threonine phosphatase, calcineurin. Calcineurin is responsible for activating NFAT by removing inhibitory phosphate groups from serine residues within the NFAT regulatory domain, thus exposing a nuclear localization sequence. Interestingly, BTPs do not inhibit the phosphatase activity of calcineurin *in vitro* (Djuric et al., 2000b). Thus BTPs represent a unique class of immuno-suppressant compounds. It has since been determined that BTPs prevent NFAT activation by blocking store-operated calcium entry, via an unknown mechanism (Ishikawa et al., 2003; Zitt et al., 2004). We have confirmed this finding and, utilizing an affinity purification approach, have identified the actin reorganizing protein drebrin as a likely target of BTP. Drebrin is a member of the ADF-H/cofilin family of actin-binding proteins and has been implicated in actin rearrangements driving dendritic spine outgrowth in neurons (Hayashi et al., 1996; Hayashi and Shirao, 1999; Ishikawa et al., 1994; Sasaki et al., 1996; Shirao et al., 1994; Takahashi et al., 2003; Toda et al., 1999a). We show that BTP is able to block drebrin dependent actin rearrangement. We also demonstrate that drebrin expression is essential for activation of store-operated calcium entry in Jurkat T cells, as reduction in drebrin protein expression by siRNA treatment results in a block in store-operated channel mediated $[Ca^{2+}]_i$ increase but not ER Ca^{2+} release, similar to that seen with BTP treatment. Together, these data indicate that BTP blocks store-operated channel activation by binding to the actin-binding protein drebrin, which plays an essential role in store-operated channel activation.

2. Experimental procedures

2.1. Cells, antibodies, plasmids, and reagents

Jurkat E6-1 T cells were grown in complete RPMI supplemented with 5% FCS. HEK293T and CHO cells were grown in complete DMEM supplemented with 5% FCS. Anti-drebrin antibody was from Sigma (St. Louis, MO), anti-GFP and anti-actin antibodies from Santa Cruz Biotech (Santa Cruz, CA). Alexa-fluor 568 conjugated phalloidin was from Molecular Probes (Eugene, OR). Drebrin mutants R236M237M (R mutant), K270MK271M (K mutant) and Q297LQ298L (Q mutant) were generated by standard molecular biology techniques. BTP (N-[4-[3,5-bis(trifluoromethyl)-1H-pyrazole-1-yl] phenyl]-4-methyl-1,2,3-thiadiazol) was synthesized as previously described as well as provided as a kind gift of Drs. James Trevillyan and Stevan Djuric, Abbott Laboratories, Chicago, IL (Djuric et al., 2000a). Synthesis of other BTP compounds is described in supplementary Fig. 1.

2.2. Fluorescent calcium measurement

Changes in $[Ca^{2+}]_i$ were measured by loading Jurkat cells with 1 μM Fura-2AM (Sigma, St. Louis, MO) as described in (Hao et al., 2003) except that cells were loaded and assayed in Ringer's solution (155 mM NaCl, 4.5 mM KCl, 2 mM $MgCl_2$, 10 mM dextrose, 5 mM HEPES, pH 7.4). Cells (1×10^6/ml in 1 ml) were loaded in the presence of 1 mM $CaCl_2$ and washed with Ringer's solution without $CaCl_2$ prior to assay. For BTP treatment, cells were treated for 1 h with 1 μM BTP and loaded with Fura-2AM for the final 30 min of treatment.

2.3. Transfections and analysis of transfected cells

CHO and 293T cells were grown on glass coverslips, then transfected, followed by analysis 24 to 48 h later. Cells were imaged live, or fixed for 15 min in PBS containing 4% para-formaldehyde and permeabilized with PBS containing 1% Triton-X 100 for 2 min. Cells were then blocked in PBS containing 5% BSA. Cells were stained with Alexa-568 phalloidin (Molecular Probes, Eugene, OR) to visualize F-actin. Cells were then analyzed on an Olympus Fluoview 300 confocal laser scanning microscope (Olympus Microscope, Melville, NY). Images were analyzed using ImagePro.

2.4. siRNA and shRNA knockdown

Drebrin expression was knocked down by transfecting 2.0×10^7 Jurkat cells with 200 nM drebrin-specific siRNAs (catalog # 011841, accession #s: NM_004395 and NM_080881) or 200 nM siControl #1 non-targeting control siRNAs (Smartpool, Dharmacon, LaFayette, CO). siRNAs were transfected by electroporation using a BTX electrosquare porator 800 (Genetronics, San Diego, CA) at 300 V for 20 ms in 400 μl RPMI in a 4 mm electroporation cuvette. Cells were then cultured in RPMI-C + 10% FCS for 48–96 h prior to assay. Cells were then screened for expression of drebrin by Western blot.

2.5. In-gel digest and mass spectrometry

For protein identification, bands were excised from the gel and in-gel tryptic digest was performed following kit instructions