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

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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.

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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$_3$R) 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

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
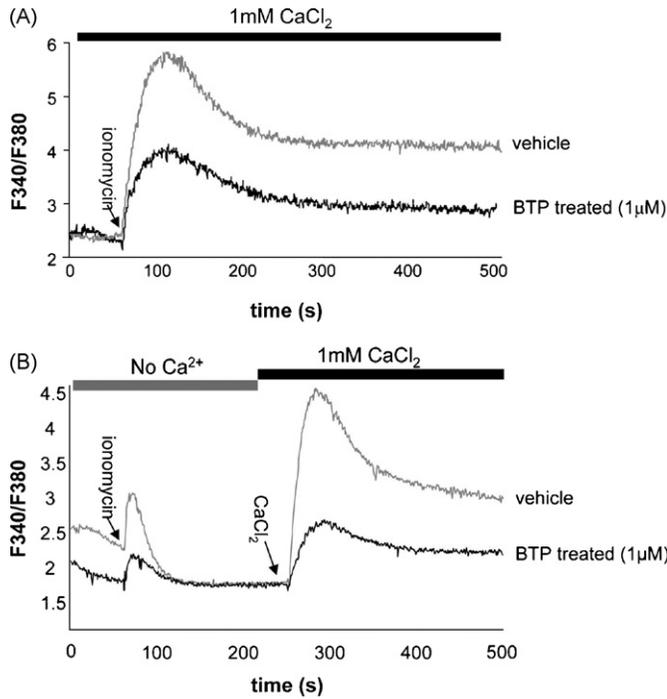
Fig. 1. BTP blocks intracellular calcium mobilization. Jurkat T cells were treated with DMSO or BTP and loaded with Fura-2AM in order to measure intracellular calcium concentration. (A) Cells were stimulated with ionomycin in Ringer's solution in the presence of 1 mM extracellular calcium. One of at least 5 experiments. (B) Cells were stimulated with ionomycin in Ringer's solution without extracellular calcium. 1 mM CaCl$_2$ was added as indicated and calcium influx monitored. 1 of 3 experiments.

(In-gel digestion kit, Pierce Biotech Inc., Rockford, IL) prior to submission to The Proteomics and Mass Spectrometry Core Facility at Penn State University (University Park, PA) for mass spec analysis. Alternatively, excised bands were sent to W.M. Keck Foundation Biotechnology Resource Laboratory at Yale University (New Haven, CT) for in-gel digestion and mass spec analysis. Peptide masses were used to search either the ProFound or Mascot databases as indicated for matching proteins (Zhang and Chait, 2000; Perkins et al., 1999).

3. Results

3.1. Drebrin is a BTP binding protein

As previously reported by others, when we treated Jurkat T cells with BTP2 prior to stimulation with ionomycin, which triggers an increase in [Ca^{2+}]$_i$, we observed inhibition of this response (Fig. 1A) (Zitt et al., 2004; Ishikawa et al., 2003; He et al., 2005). Acute addition of BTP to cells once calcium increase has been induced results in an immediate reduction in calcium influx (Supplementary Fig. 1). Using a calcium add back assay where cells were initially stimulated in the absence of extracellular Ca^{2+} and then Ca^{2+} restored to the buffer following the return of [Ca^{2+}]$_i$ to baseline, we find that BTP pretreatment results in a significant decrease in Ca^{2+} entry following addition of extracellular Ca^{2+}. By contrast the initial store release was not consistently affected, indicating that the blunted calcium response caused by BTP is due to inhibition of Ca^{2+} entry and not Ca^{2+} store release (Fig. 1B). The ability of BTP to inhibit calcium entry was also observed when cells were treated with the SERCA pump inhibitor thapsigargin (Supplementary Fig. 1).

In order to better understand the mechanism by which BTP inhibits store-operated channels, we set up an affinity purification system to purify and identify BTP binding proteins. A derivative of BTP1 was synthesized coupled to biotin as described in supple-
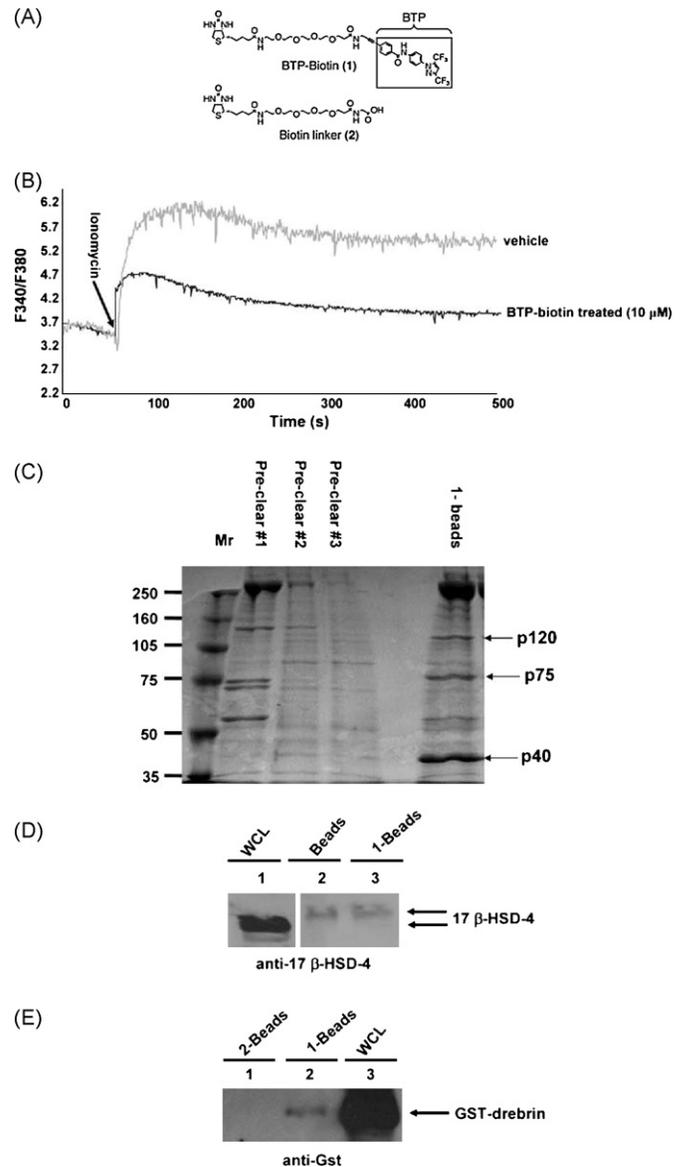

Fig. 2. Identification of BTP binding proteins using a modified BTP affinity probe. (A) Structure of BTP-biotin (1) and biotin-linker (2). (B) Jurkat T cells were pretreated with 10 μM biotinylated BTP or DMSO and loaded with Fura-2AM prior to stimulation with ionomycin in the presence of extracellular calcium. 1 of 3 experiments. (C) Jurkat T-cell lysate was incubated with the SA-agarose beads alone 3 times (preclear # 1, 2 and 3), followed by SA-agarose beads coated with (1). Beads were washed extensively, then boiled in SDS-PAGE reducing buffer prior to SDS-PAGE and staining using Coumassie blue stain to identify binding proteins. Arrows indicate p120 (identified as drebrin), p75 (identified as 17-β-HSD4) and p40 (identified as actin). (D) Jurkat T-cell lysate was incubated with SA-agarose beads alone or SA-beads coated with (1), washed extensively, then boiled in SDS-PAGE reducing buffer prior to SDS-PAGE and Western blotting with anti-17-β-HSD4. Lane 1: WCL of Jurkat T cells; lane 2: pull-down with SA-beads alone; lane 3: pull-down with SA-beads coated with (1). Blot probed with anti-17-β-HSD4. (E) Lysates from *E. coli* expressing Gst-drebrin were incubated with SA-agarose beads coated with (2) or SA-beads coated with (1), washed extensively, then boiled in SDS-PAGE reducing buffer prior to SDS-PAGE and Western blotting with Gst. Lane 1: pulldown with SA-beads coated with (2); lane 2: pulldown with SA-beads coated with (1); lane 3: WCL of Jurkat T cells. Blot probed with anti-Gst.

mentary methods and purified to greater than 99% purity (Fig. 2A, see supplementary methods). This biotinylated BTP compound was then tested for its ability to prevent NFAT activation in primary thymocytes carrying a transgenic NFAT luciferase reporter. Addition of the large linker and biotin groups reduced the potency of the biotinylated BTP compared with BTP, however, it still had signifi-

cant activity in these cells (IC$_{50}$ ∼ 600 nM compared to ∼15 nM for parent compound, data not shown) (Zitt et al., 2004). Additionally, at 10 μM, biotinylated BTP was able to inhibit Ca^{2+} mobilization in response to ionomycin treatment (Fig. 2B). Biotinylated BTP was immobilized onto streptavidin-coated agarose beads and used to purify BTP binding proteins from Jurkat T cell lysates following pre-clearing with beads coated with the biotinylated linker (Fig. 2A). Purified proteins were then separated by SDS-PAGE and visualized with Coumassie stain (Fig. 2C). Unique bands from biotinylated BTP coated beads were excised from the gel and subjected to in-gel tryptic digestion and MALDI/TOF mass spectrometry based protein identification. This procedure was performed twice and the samples were sent to two different facilities for mass spectrometric analysis. Both facilities identified p120 as the actin-binding protein drebrin (Z score 2.32, 99.0 percentile, probability of a match = 1.0e + 000 using ProFound in experiment 1, and p < 0.05 using Matrixscience in experiment 2, supplementary Tables 1 and 2). Drebrin has been well studied in neuronal cells and appears to be important for actin rearrangements such as those driving neuronal dendritic spine outgrowth (Takahashi et al., 2003; Toda et al., 1999b; Hayashi and Shirao, 1999). Binding of drebrin to BTP was confirmed by performing pull-down assays using the biotinylated BTP coupled to streptavidin-coated agarose beads, followed by Western blot to detect endogenous drebrin (Fig. 3A). The interaction was further confirmed by over-expressing a GFP-drebrin fusion protein in HEK293T cells and performing the pull-down experiment with lysates from these cells prior to Western blotting to detect the GFP tag (Fig. 3B) (Hayashi et al., 1999). The specificity of the drebrin/BTP interaction was confirmed by performing the pull-down assay with streptavidin-agarose beads coated with the biotin-linker compound without the BTP moiety (2) (Fig. 3B and C). In addition, soluble BTP competed with the biotinylated BTP for binding to drebrin, and we estimate the Kd to be in the low nM range (<300 nM based on preliminary binding experiments) (Fig. 3C). We confirmed that drebrin directly interacts with BTP using bacterially expressed Gst-drebrin (Fig. 2E). We also sequenced and identified p75 as 17 β-hydroxysteroid dehydrogenase IV, and p40 as actin. We ruled out 17 β-hydroxysteroid dehydrogenase IV as a bona fide BTP binding protein as it interacted non-specifically with the SA-beads used for the pull-downs (Fig. 2D). The identification of actin along with drebrin is not unexpected as drebrin has been demonstrated to interact with and co-immunoprecipitate with actin (Fucini et al., 2000, 2002; Hayashi et al., 1999).

3.2. K270 and K271 within drebrin are important for BTP binding

Analysis of fragments of drebrin for binding to the biotinylated BTP coupled to streptavidin-coated agarose beads indicated that the full-length protein had the highest binding capacity, and the N-terminal 1–366 amino acids retained strong binding (Fig. 3D). This region includes the ADF-H and actin-binding domains, but lacks the proline rich region of drebrin (Hayashi and Shirao, 1999). Further analysis indicated that amino acids 233–366 of drebrin maintained minimal binding, however this binding was much below that observed in the full-length or N-terminal 366 amino acids (Fig. 3D). This latter region includes the full actin-binding domain and a small region C-terminal to this region (compare to fragment containing amino acids 233–317, which contains just the actin-binding domain). Thus the interaction between BTP and drebrin includes the actin-binding domain, but requires residues that flank this region at the N- and C-termini of this domain for optimal binding. To identify residues within this region of drebrin that interacts with BTP, we focused on highly charged residues within the central actin-binding domain, and generated several mutants, one of which K270MK271M, destroyed binding to drebrin, while other closely
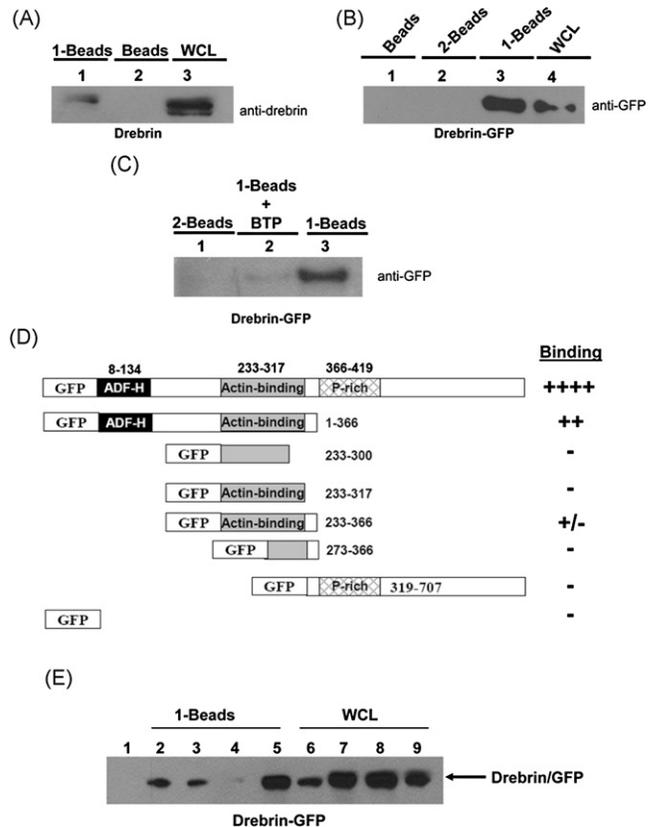

Fig. 3. BTP binds to drebrin. (A) Jurkat T-cell lysate was incubated with the indicated coating of SA-agarose beads. Beads were boiled in SDS-PAGE reducing buffer prior to SDS-PAGE and Western blotting. Lane 1: biotinylated BTP; lane 2: DMSO; lane 3, 5% of the input. Blot was probed using anti-drebrin antibody. 1 of 3 similar experiments. (B) CHO cells were transfected with GFP-drebrin plasmid. Cells were then lysed and lysates incubated with the indicated coating of SA-agarose beads as in (A). Lane 1: DMSO; lane 2: biotinylated linker; lane 3: biotinylated BTP; lane 4: 5% of the input. Blot was probed using anti-GFP antibody. 1 of 3 similar experiments. (C) Lysates from CHO cells transfected with GFP-drebrin incubated as follows: lane 1: 2-SA-beads; lane 2: biotinylated BTP-SA-beads along with 10 μM BTP; or lane 3: biotinylated BTP-SA-beads alone. Blot was probed using anti-GFP antibody. 1 of 3 similar experiments. (D) HEK293T cells were transfected with plasmids encoding the indicated fragments of drebrin fused to GFP. Cells were lysed and lysates were incubated with SA-beads alone, biotinylated linker-SA-beads, or biotinylated BTP-SA-beads, washed, separated by SDS-PAGE and probed with anti-GFP. None of the fragments associated with either the SA-beads or biotinylated linker-SA-beads alone. Relative binding of each drebrin fragment to BTP-biotin compared to full-length drebrin with (++++) representing binding the full-length drebrin and (−) representing no binding observed. 1 of 2 similar experiments. (E) Lysates from CHO cells transfected with WT GFP-drebrin incubated with SA-beads alone (lane 1) or WT or the indicated mutants of drebrin incubated with 1-SA-beads as follows: lane 2: WT; lane 3: R236,237 mutant; lane 4: K270,271 mutant; lane 5: Q297,298 mutant. Lanes 6–9 were loaded with lysates from cells expressing WT, R, K and Q mutants respectively. Blot was probed using anti-GFP antibody. 1 of 2 similar experiments.

located mutants (R236MR237M, Q297LQ298L) retained binding (Fig. 3E, data not shown).

3.3. BTP blocks drebrin function

When over-expressed in fibroblasts drebrin causes the formation of long, branched extension and curved, thick actin bundles (Shirao et al., 1994). In order to assess drebrin as a target of BTP, GFP-tagged drebrin was over-expressed in CHO cells, which caused cells to develop long filopodia-like membrane extensions (FLE) that were highly branched, with drebrin co-localized with actin (Fig. 4). When drebrin over-expressing cells were treated with BTP (or biotinylated BTP, data not shown), there was a drastic reduction in filopodia-like extensions (FLE) and in the number of branch points
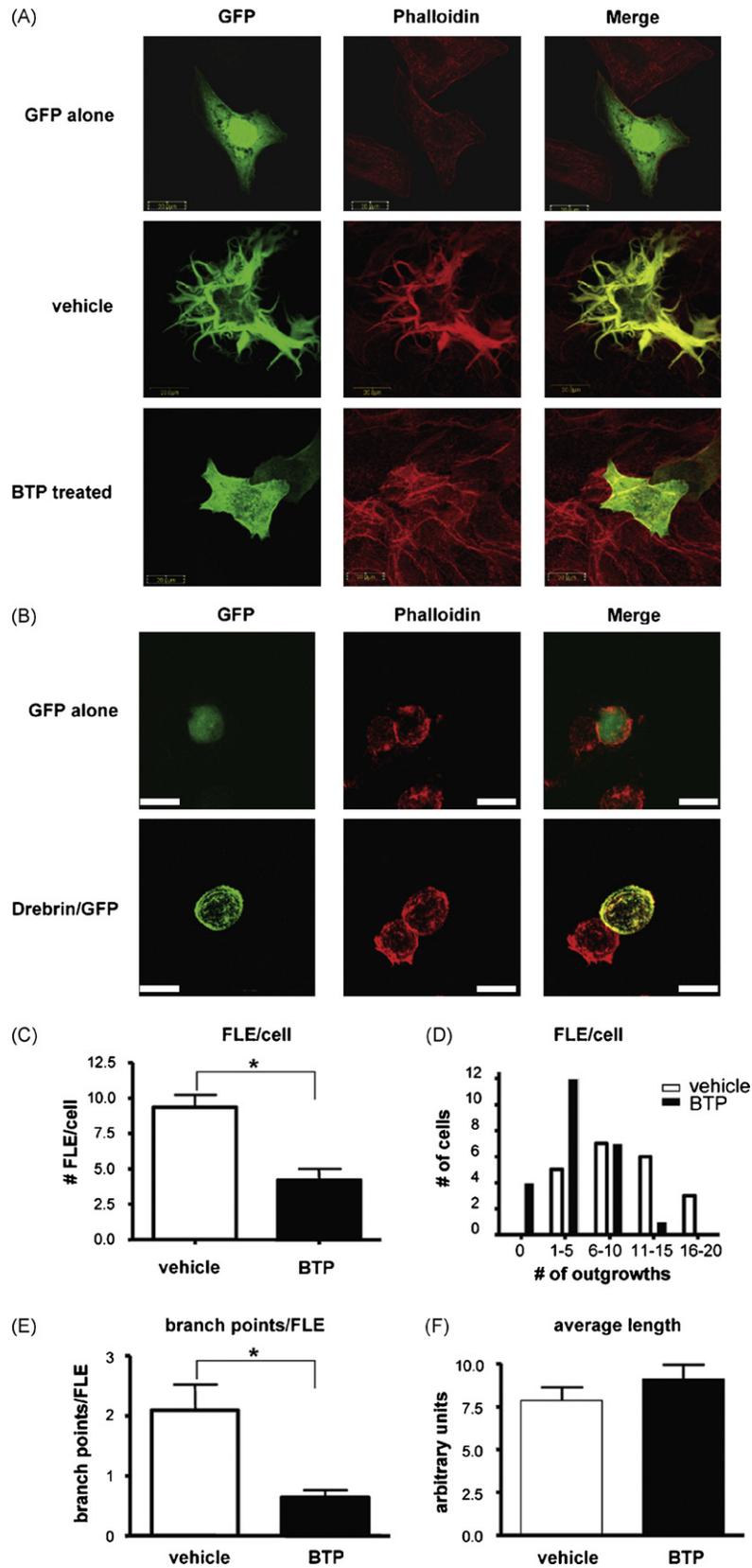
Fig. 4. BTP inhibits drebrin function. (A) CHO cells transfected with either GFP (top panel) or GFP-drebrin (middle and bottom panels). Cells were treated with DMSO (vehicle, middle panel) or BTP (bottom panel) prior to staining for F-actin (red) and visualization using confocal laser scanning microscopy. 1 of 3 similar experiments. (B) Jurkat T cells were transfected with either GFP (top panel) or GFP-drebrin (bottom panel). Cells were stained for F-actin (red) and visualized as in (A). 1 of at least 5 similar experiments. White bar indicate 20 μm. (C) Branched cell extensions caused by drebrin overexpression (filopodia-like extensions, FLE) were counted on each cell in DMSO and BTP treated cells and expressed as average FLE per cell, (D) the number of cells having a given range of FLEs per cell, (E) the average number of branch points per FLE, and (F) the average length of each FLE. For all measurements $n = 25$ cells, $*p < 0.05$. 1 of 3 similar experiments.
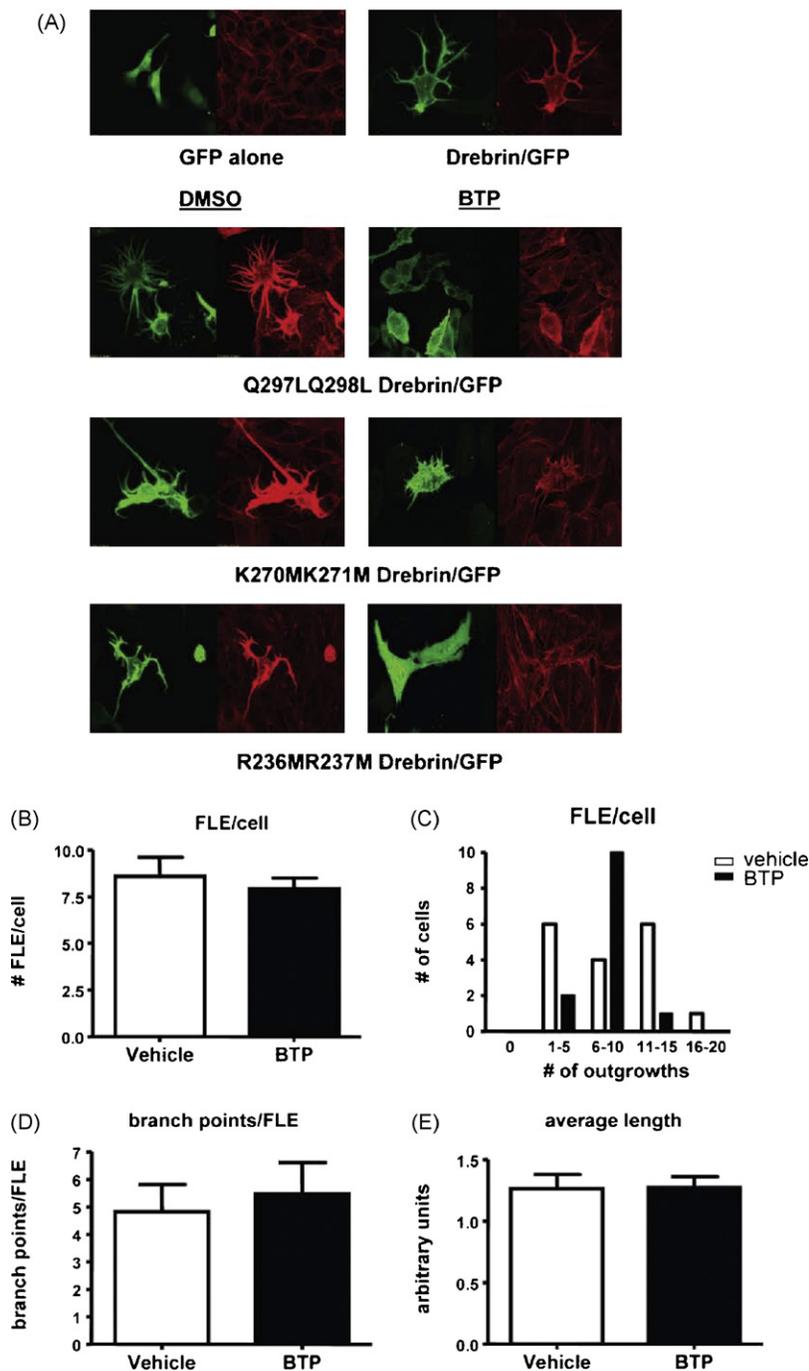
Fig. 5. BTP binding mutant of drebrin retains actin reorganization function. (A) CHO cells transfected with the indicated mutants of drebrin fused to GFP. Left panels show GFP and right panels show F-actin staining. (B–E) CHO cells transfected with K270MK271M mutant of drebrin fused to GFP. 1 of 3 similar experiments. (B) Branched cell extensions caused by drebrin overexpression (filopodia-like extensions, FLE) were counted on each cell in DMSO and BTP treated cells and expressed as average FLE per cell, (C) the number of cells having a given range of FLEs per cell, (D) the average number of branch points per FLE, and (E) the average length of each FLE. For all measurements $n = 17$ for vehicle, $n = 13$ for BTP treated cells. Images were analyzed as in Fig. 3. 1 of 2 similar experiments.

formed per each FLE but not in the average length of these processes (Fig. 4A and C–F). Thus, BTP treated cells had fewer FLE, and the FLE that formed were long and linear as opposed to branched, indicating that BTP affects drebrin's ability to induce plasticity in the actin cytoskeleton. However, drebrin co-localization with actin was not apparently affected, suggesting that although the actin-binding site within drebrin forms part of the BTP binding domain, this may not affect actin co-localization with drebrin (Figs. 4A and 2C). Overexpression of drebrin in Jurkat T cells did not lead to the development of FLEs, suggesting that drebrin only affected actin superstructure in adherent cells such as CHO cells (Fig. 4B). Of interest, the drebrin mutants R236MR237M, Q297LQ298L, as well as the K270MK271M mutant that lost binding to BTP all retained actin localization and reorganization activity, suggesting that these residues are not critical for this function of drebrin (Fig. 5A). Analysis of the K270MK271M mutant of drebrin indicated that BTP no longer inhibited FLEs in cells expressing this mutant (Fig. 5B–E).
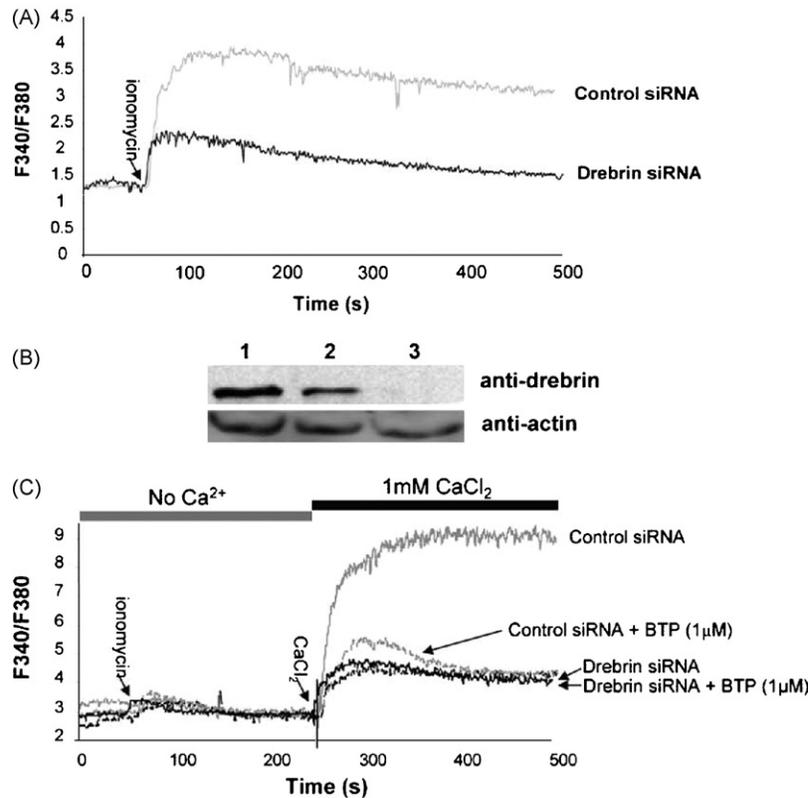
Fig. 6. Loss of drebrin expression prevents extracellular calcium influx. (A) Jurkat T cells were transfected with either control siRNAs or drebrin-specific siRNAs and grown for 48 h following transfection. Cells were then loaded with Fura-2AM and intracellular calcium concentration monitored in the presence of 1 mM extracellular Ca^{2+} before and after ionomycin treatment. 1 of 3 similar experiments. (B) Representative drebrin knockdown 48 h post-transfection in Jurkat T cells. Lane 1: Jurkat lysate no siRNA; lane 2: Jurkat lysates from cells treated with control siRNA; or lane 3: Jurkat lysates from cells treated with drebrin-specific siRNA. 1 of 3 similar experiments. (C) Jurkat T cells were transfected with either control or drebrin-specific siRNA. 48 h post-transfection, cells were treated with DMSO or 1 μM BTP and then loaded with Fura-2AM. Intracellular calcium concentration was monitored following initial stimulation with ionomycin in the absence of extracellular calcium. After $[Ca^{2+}]_i$ returned to baseline levels, 1 mM $CaCl_2$ was added and calcium influx determined by monitoring the change in Fura-2 fluorescence. 1 of 3 similar experiments.

3.4. Drebrin expression is required for calcium influx into cells

Since actin rearrangement has previously been linked to store-operated channel regulation we were interested in determining if this actin reorganizing protein was important for store-operated channel operation (Patterson et al., 1999; Hao and August, 2005a). When we used drebrin-specific siRNA to reduce drebrin protein expression in Jurkat T cells, we observed a reduction in $[Ca^{2+}]_i$ increase following ionomycin treatment, which was not observed in cells transfected with control siRNA (Fig. 6A). This defect was a result of reduced drebrin expression and not the transfection procedure itself as the drebrin-specific siRNA significantly reduced the amount of drebrin protein whereas control siRNA had much less of an effect on the protein levels of drebrin (Fig. 6B). Drebrin knockdown appears to inhibit only store-operated channel mediated Ca^{2+} entry, as when these cells are stimulated in Ca^{2+} free buffer we continued to see an initial spike in Ca^{2+} corresponding to intracellular stores (Fig. 6C). However, when we added extracellular calcium we observed only a slight increase in $[Ca^{2+}]_i$ in cells transfected with the drebrin siRNA compared with control siRNA transfected cells (Fig. 6C). We treated the drebrin-specific RNA transfected cells with BTP prior to performing the Ca^{2+} add back assay and found no further reduction in Ca^{2+} entry indicating that BTP does not act on a separate pathway and further suggesting that drebrin is the target of BTP in regulating Ca^{2+} entry (Fig. 6C). By contrast, overexpression of drebrin did not affect calcium influx, suggesting that drebrin expression is not limiting (supplementary Fig. 2A and B).

4. Discussion

The small-molecule immuno-suppressant BTP has recently been shown to block activation of store-operated calcium channels (He et al., 2005). In this study, we have identified the actin-binding protein drebrin as a BTP binding protein, and shown that reduction in drebrin expression results in similar effects to that seen with BTP treatment. Using inhibitors of actin polymerization/depolymerization, others have established a link between cytoskeletal rearrangement and store-operated channel regulation or calcium influx into cells (Patterson et al., 1999; Hao and August, 2005a). Specifically, following intracellular store depletion, actin filaments must be depolymerized and then repolymerized in order to activate store-operated channels. In DT40 B cells, treatment with low concentrations of actin depolymerizing agents such as latrunculin B caused sustained activation of Ca^{2+} influx (Hao and August, 2005a). These concentrations used are thought to be low enough so as to favor actin depolymerization, but not fully prevent repolymerization, thus the net effect is to increase actin filament turn-over (Hao and August, 2005a). Thus increasing the plasticity of the actin cytoskeleton seems to favor store-operated channel activation. Although a clear role for actin rearrangement has been established, few actin-regulating proteins have been implicated in this process. Recently, the actin modulating protein WAVE2 was shown to modulate store-operated channel in T cells, further supporting the idea that modulating the actin cytoskeleton modulate store-operated channel and calcium influx (Nolz et al., 2006). Similar to our work with drebrin, reduction in the expression of WAVE2

results in selective block in calcium influx into cells. Therefore, our data indicating that drebrin is involved in store-operated channel regulation should provide further insight into the relationship between the cytoskeleton and store-operated channel regulation.

The identity of proteins that make up the store-operated channels have recently been revealed with the cloning and characterization of Orai, along with STIM1, which seems to act as a modulator of Orai's function (Mercer et al., 2006; Soboloff et al., 2006; Peinelt et al., 2006; Zhang et al., 2005; Liou et al., 2005; Roos et al., 2005; Yeromin et al., 2006; Prakriya et al., 2006). STIM1 seems to function by translocating near to or into the plasma membrane, and seems to provide a link between the ER, where calcium is released, to the plasma membrane, where Orai acts as a channel to allow calcium into the cell (Hauser and Tsien, 2007; Ross et al., 2007; Wu et al., 2006; Spassova et al., 2006; Zhang et al., 2005). Of interest is the observation that STIM1 can interact with TRPC proteins, as well as Orai, and TRPC proteins may modulate calcium channel activity (Liao et al., 2007; Ong et al., 2007; Huang et al., 2006; Lopez et al., 2006).

Drebrin may regulate calcium entry by facilitating actin rearrangement in association with store-operated channels following store depletion. This may be mediated by formation of a complex at the IP$_3$R where drebrin could interact with homer adaptor proteins, which have been shown to bind both IP$_3$R and drebrin, as well as the TRPC family of ion-channels (Yuan et al., 2003; Shiraishi et al., 1999). Drebrin has also recently been shown to exist in a complex and co-immunoprecipitate with TRPC5 and TRPC6 (Goel et al., 2005). In addition, it was recently reported that BTP2 modulates the function of TRPM4, which has been shown to modulate store-operated channel (Takezawa et al., 2006). By recruiting drebrin to such complexes, the associated actin structure could be induced to rearrange, thus regulating store-operated channel function. Further research dissecting multi-protein complexes that drebrin participates in may lead to a better understanding of the mechanisms regulating store-operated channel function.

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biocel.2009.11.019.

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