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Original Paper

Lithium Sensitivity of Store Operated Ca^{2+} Entry and Survival of Fibroblasts Isolated from Chorea-Acanthocytosis Patients

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Key Words
Calcium • SOCE • Lithium • Apoptosis • Neurodegeneration • Orai1 • Cell membrane scrambling

Abstract
Background: The widely expressed protein chorein fosters activation of the phosphoinositide 3 kinase (PI3K) pathway thus supporting cell survival. Loss of function mutations of the chorein encoding gene VPS13A (vacuolar protein sorting-associated protein 13A) causes chorea-acanthocytosis (ChAc), a neurodegenerative disorder paralleled by deformations of erythrocytes. In mice, genetic knockout of chorein leads to enhanced neuronal apoptosis. PI3K dependent signalling upregulates Orai1, a pore forming channel protein accomplishing store operated Ca^{2+} entry (SOCE). Increased Orai1 expression and SOCE have been shown to confer survival of tumor cells. SOCE could be up-regulated by lithium. The present study explored, whether SOCE and/or apoptosis are altered in ChAc fibroblasts and could be modified by lithium treatment. ***Methods:*** Fibroblasts were isolated from ChAc patients and age-matched healthy volunteers. Cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$) was estimated from Fura-2-fluorescence, SOCE from increase of $[Ca^{2+}]_i$ following Ca^{2+} re-addition after Ca^{2+}-store depletion with sarcoendoplasmatic Ca^{2+}-ATPase (SERCA) inhibitor thapsigargin (1 μM), and apoptosis from annexin-V/propidium iodide staining quantified in flow cytometry. ***Results:*** SOCE was significantly smaller in ChAc fibroblasts than in control fibroblasts. Lithium (2 mM, 24 hours)

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significantly increased and Orai1 blocker 2-Aminoethoxydiphenyl Borate (2-APB, 50 μM, 24 hours) significantly decreased SOCE. Annexin-V-binding and propidium iodide staining were significantly higher in ChAc fibroblasts than in control fibroblasts. In ChAc fibroblasts annexin-V-binding and propidium iodide staining were significantly decreased by lithium treatment, significantly increased by 2-APB and virtually lithium insensitive in the presence of 2-APB. ***Conclusions:*** In ChAc fibroblasts, downregulation of SOCE contributes to enhanced susceptibility to apoptosis. Both, decreased SOCE and enhanced apoptosis of ChAc fibroblasts can be reversed by lithium treatment.

Introduction

Chorein supports the activation of phosphoinositide-3-kinase (PI3K)-p85-subunit with subsequent activation of ras-related C3 botulinum toxin substrate 1 (Rac1) and p21 protein-activated kinase 1 (PAK), thus fostering actin polymerization and cell survival [1-4]. Loss-of-function mutations of the chorein encoding gene VPS13A (vacuolar protein sorting-associated protein 13A) cause chorea-acanthocytosis (CA) [5-9], characterized by progressive hyperkinetic movement disorder, cognitive dysfunction, behavioural abnormalities, epilepsy, increased plasma creatine kinase concentration, and erythrocyte acanthocytosis [5, 10]. Gene targeted mice lacking functional chorein display erythrocyte shape changes [11], neuronal apoptosis [12] and altered behaviour [12].

Chorein is widely expressed [13-15] and contributes to regulation of platelet activation [15], endothelial cell stiffness [14] and tumor cell survival [4]. Chorein is particularly important for function and survival of neurons and skeletal muscle cells [5, 10]. In platelets, chorein deficiency decreases the number of intracellular granules and compromises platelet degranulation [15]. Chorein deficiency further compromizes dopamine release in pheochromocytoma (PC12) cells [16, 17]. In fibroblasts isolated from ChAc-patients cytoskeletal architecture is severely distorted [17].

In rhabdomyosarcoma cells, chorein has been shown to up-regulate store operated Ca^{2+} entry [18] which is accomplished by the pore-forming Ca^{2+} channel subunits Orai1, Orai2 and/or Orai3 [19-23] as well as their regulators STIM1 and/or STIM2 [24-28]. Increases of cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$) are powerful regulators of cell survival and cell death [29-33]. In bone cells, Orai1 and thus SOCE is up-regulated by lithium [34], which favorably influences the course of neurodegenerative disease [35-42].

The present study explored whether ChAc fibroblasts differ from fibroblasts of healthy individuals in SOCE and apoptosis and whether SOCE and apoptosis of ChAc fibroblasts are sensitive to lithium treatment.

Materials and Methods

Cells

The study has been approved by the Institutional Review Board of the Technische Universität Dresden (EK45022009). Fibroblasts from patients (n=5) and healthy volunteers (n=5) were isolated from human skin biopsy after written consent by the patients and controls. Skin biopsies were minced using sterile techniques and washed twice in phosphate buffered saline (PBS) supplemented with antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin). Explants were placed into 25 cm^2 flasks and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and antibiotics, as described above. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Once the fibroblasts were established, the fungizone was omitted from the medium. Confluent cells were detached by treatment with 0.25% trypsin and 0.05% EDTA for 5 min, and aliquots of separated cells were subcultured with the same medium. Cell cultures between the third and twelfth passages were used in this study [17].

Western Blotting

Orai1 protein abundance was quantified by western blotting. Cells were washed twice with ice cold PBS and suspended in 200 µl ice-cold RIPA lysis buffer (Thermo Fisher Scientific, USA) containing Halt Protease and Halt Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific, USA). Protein concentration was determined using the Bradford assay (BioRad, München, Germany). Fifty - hundred µg of protein were solubilized in sample buffer at 95ºC for 5 min. The proteins were separated by a 12% SDS-PAGE in a Glycine-Tris buffer and electro-transferred into Nitrocellulose membranes for 70 min. After blocking with 5% milk in TBST at room temperature for 1-2 h, the membranes were incubated with primary anti-Orai1 antibody (1:1000, Proteintec) and anti-GAPDH antibody (1:2000, Cell Signaling) at 4°C overnight. After washing (TBST), the blots were incubated with secondary anti-rabbit (1:2000, Cell Signaling) antibody for 1 h at room temperature. Protein bands were detected after additional washes (TBST) with an ECL detection reagent (Amersham, Freiburg, Germany) and quantified with Quantity One Software (BioRad, München, Germany). To assign the right protein size we used Protein-Marker VI (Peqlab, Erlangen, Germany).

Ca^{2+} measurements

Fura-2 fluorescence was utilized to determine intracellular Ca^{2+} measurements [43]. Cells were loaded with Fura-2/AM (2 µM, Invitrogen, Goettingen, Germany) for 20 min at 37°C. Cells were excited alternatively at 340 nm and 380 nm through an objective (Fluor 40×/1.30 oil) built in an inverted fluorescence microscope (Axiovert 100, Zeiss, Oberkochen, Germany). Emitted fluorescence intensity was recorded at 505 nm. Data were acquired using specialized computer software (Metafluor, Universal Imaging, Downingtown, USA). Cytosolic Ca^{2+} activity was estimated from the 340 nm/380 nm ratio. SOCE was determined by extracellular Ca^{2+} removal and subsequent Ca^{2+} re-addition in the presence of thapsigargin (1 µM, Invitrogen). For quantification of Ca^{2+} entry, the slope (delta ratio/s) and peak (delta ratio) were calculated following re-addition of Ca^{2+}.

Experiments were performed with Ringer solution containing (in mM): 125 NaCl, 5 KCl, 1.2 $MgSO_4$, 2 $CaCl_2$, 2 Na_2HPO_4, 32 HEPES, 5 glucose, pH 7.4. To reach nominally Ca^{2+}-free conditions, experiments were performed using Ca^{2+}-free Ringer solution containing (in mM): 125 NaCl, 5 KCl, 1.2 $MgSO_4$, 2 Na_2HPO_4, 32 HEPES, 0.5 EGTA, 5 glucose, pH 7.4.

Analysis of apoptosis

After incubation, the cells were centrifuged at 1,000 r.p.m. for 5 minutes. Then, cells were co-stained with annexin V-FITC (Immunotools, Friesoythe, Germany) to assess phosphatidylserine exposure and propidium iodide (PI, Sigma-Aldrich) to estimate cell membrane integrity. To this end cells were incubated in 100 µl Ringer solution containing 5 mM Ca^{2+} and 1 µl annexin V-FITC (Immunotools, Friesoythe, Germany), and 0.075 µl PI stock solution (25 mg/ml PI in PBS) for 15 min at 37°C. Then, 100 µl of Ringer solution were added and annexin V-FITC fluorescence as well as PI was determined by flow cytometry using a FACS calibur (BD, Heidelberg, Germany).

Statistics

Data are expressed as arithmetic means ± SEM. Statistical analysis was made by unpaired t-test or Mann-Whitney test, as appropriate. Only results with $p<0.05$ were considered statistically significant. All experiments were performed in at least 4 independent replications.

Results

The present study explored whether chorein deficiency in fibroblasts impacts on Orai1 expression and Ca^{2+} signalling. To this end, fibroblasts were isolated from healthy individuals (control fibroblasts) and from patients with chorea-acanthocytosis (ChAc fibroblasts). Orai1 expression was quantified by Western blotting. As illustrated in Fig. 1, fibroblasts from both, ChAc patients and healthy volunteers expressed Orai1. The expression was, however, significantly lower in fibroblasts from ChAc patients than in fibroblasts from healthy volunteers.

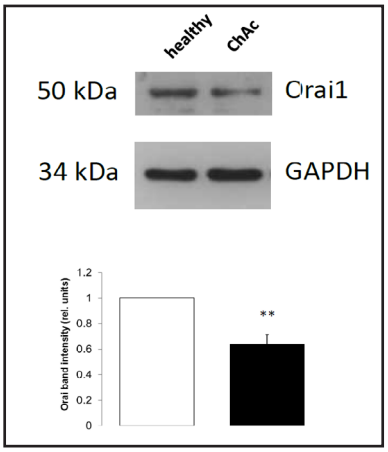

Fig. 1. Orai1 abundance in fibroblasts isolated from ChAc patients and healthy volunteers. Representative Western blot (upper panel) and arithmetic means (± SEM, n = 5 patients and 5 healthy volunteers) of Orai1 protein abundance in fibroblasts isolated from healthy volunteers (white bar) and in fibroblasts isolated from ChAc patients (black bar) ** (p<0.01) indicates statistically significant difference from healthy fibroblasts (two-tailed unpaired *t*-test).

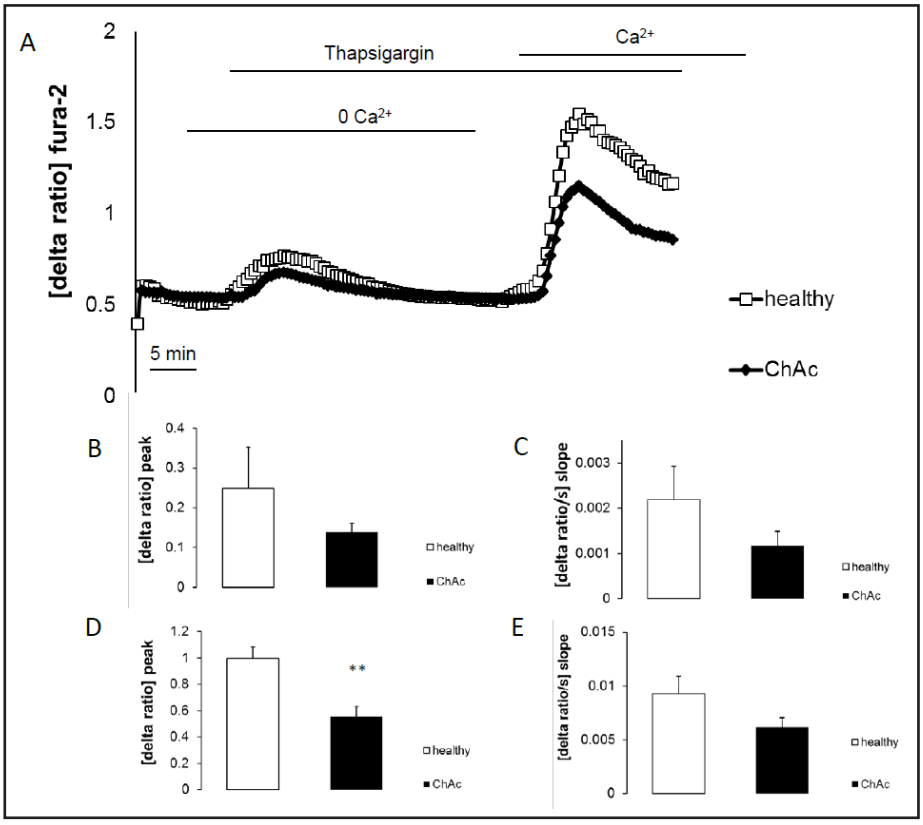

Fig. 2. Intracellular Ca^{2+} release and store-operated Ca^{2+} entry (SOCE) in fibroblasts isolated from ChAc patients and healthy volunteers. A. Representative tracings of Fura-2 fluorescence-ratio in fluorescence spectrometry before and following extracellular Ca^{2+} removal and addition of thapsigargin (1 µM), as well as re-addition of extracellular Ca^{2+} in fibroblasts isolated from healthy volunteers (white squares) and from chorea-acanthocytosis (ChAc) patients. B,C. Arithmetic means (± SEM, n = 48-61 cells from 4-5 individuals) of peak (B) and slope (C) increase of Fura-2-fluorescence-ratio following addition of thapsigargin (1 µM) in fibroblasts isolated from healthy volunteers (white bars) and in fibroblasts isolated from ChAc patients (black bars) D,E. Arithmetic means (± SEM, n = 48-61 cells from 4-5 individuals) of peak (D) and slope (E) increase of fura-2-fluorescence-ratio following re-addition of extracellular Ca^{2+} in fibroblasts isolated from healthy volunteers (white bars) and in fibroblasts isolated from ChAc patients (black bars). ** (p<0.01) indicates statistically significant difference from healthy individuals (two-tailed unpaired *t*-test).

Fura2-fluorescence was taken as a measure of cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$). In order to test whether chorein deficiency influences intracellular Ca^{2+} release and store-operated Ca^{2+} entry (SOCE), intracellular Ca^{2+} stores were depleted by exposure of the cells to Ca^{2+}-free solutions containing the sarco-/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) inhibitor thapsigargin (1 µM) followed by re-addition of extracellular Ca^{2+} in the continued presence

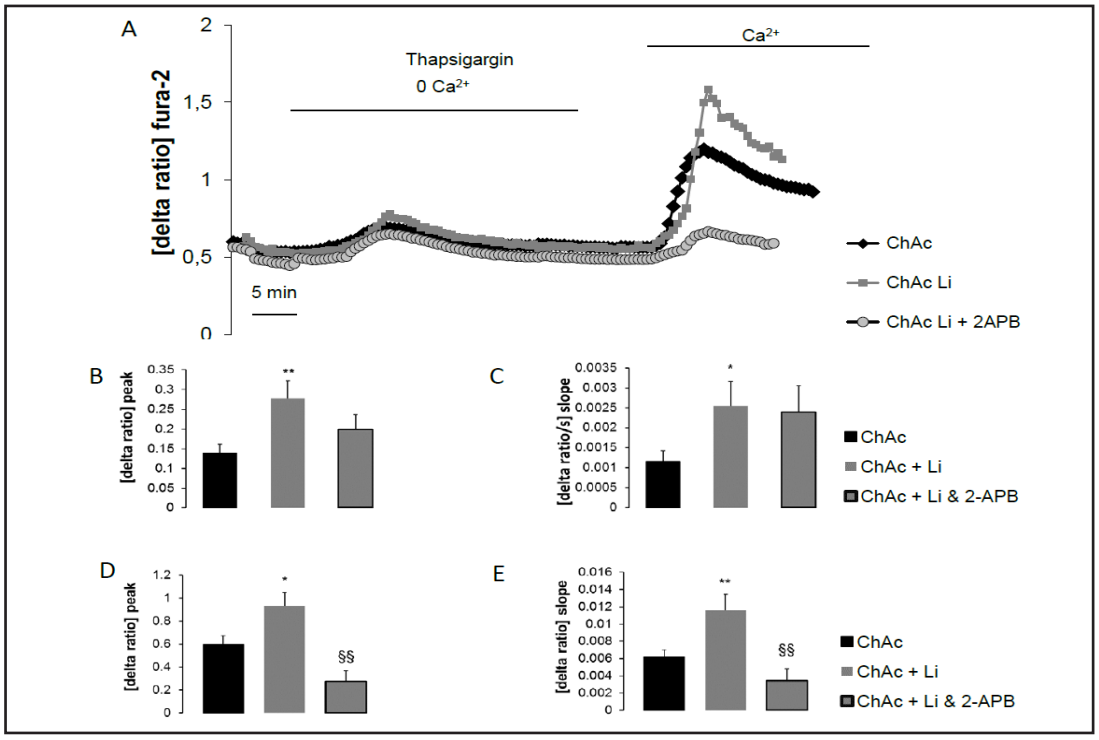

Fig. 3. Intracellular Ca^{2+} release and store-operated Ca^{2+} entry (SOCE) in fibroblasts isolated from ChAc patients with or without lithium or 2-APB treatment. A. Representative tracings of Fura-2 fluorescence-ratio in fluorescence spectrometry before and following extracellular Ca^{2+} removal and addition of thapsigargin (1 µM), as well as re-addition of extracellular Ca^{2+} in fibroblasts isolated from chorea-acanthocytosis (ChAc) patients in the continued presence of thapsigargin and absence of drugs (white circles), in the presence of lithium (black circles) and in the presence of 2-APB (grey circles). B,C. Arithmetic means (± SEM, n = 23-73 cells from 4-5 individuals) of peak (B) and slope (C) increase of fura-2-fluorescence-ratio following addition of thapsigargin (1 µM) in fibroblasts isolated from chorea-acanthocytosis (ChAc) patients in the absence of drugs (white bars), in the presence of lithium (black bars) and in the presence of 2-APB (grey bars). D,E. Arithmetic means (± SEM, n = 23-73 cells from 4-5 individuals) of peak (D) and slope (E) increase of fura-2-fluorescence-ratio following re-addition of extracellular Ca^{2+} in fibroblasts isolated from chorea-acanthocytosis (ChAc) patients in the absence of drugs (white bars), in the presence of lithium (black bars) and in the presence of 2-APB (grey bars). *($p<0.05$), ** ($p<0.01$) indicates statistically significant difference from untreated cells, §§($p<0.01$) indicates statistically significant difference to respective value in absence of 2-APB, (ANOVA).

of thapsigargin. The Fura2-fluorescence ratio prior to extracellular Ca^{2+} removal was slightly, but significantly ($p<0.05$) higher in control fibroblasts (0.64 ± 0.03, n = 6) than in ChAc fibroblasts (0.54 ± 0.02, n = 5). As illustrated in Fig. 2, the addition of thapsigargin triggered Ca^{2+} release from intracellular stores, leading to a rapid, transient increase in $[Ca^{2+}]_i$. The increase of intracellular Ca^{2+} activity following thapsigargin treatment was similar in control fibroblasts and ChAc fibroblasts (Fig. 2). The addition of extracellular Ca^{2+} in the continued presence of thapsigargin was followed by a rapid increase of Fura2-fluorescence in both, control fibroblasts and ChAc fibroblasts, reflecting store operated Ca^{2+} entry (SOCE). The peak of SOCE was significantly lower in ChAc fibroblasts than in control fibroblasts (Fig. 1).

A further series of experiments explored whether SOCE could be restored by lithium treatment (2 mM, 24 hours). Again, intracellular Ca^{2+} stores were depleted by exposure of the cells to Ca^{2+}-free solutions containing the sarco-/endoplasmic reticulum Ca^{2+}/ATPase (SERCA) inhibitor thapsigargin (1 µM) followed by re-addition of extracellular Ca^{2+} in the continued presence of thapsigargin. As shown in Fig. 3, the increase of intracellular Ca^{2+} activity following thapsigargin treatment was significantly higher in lithium treated than

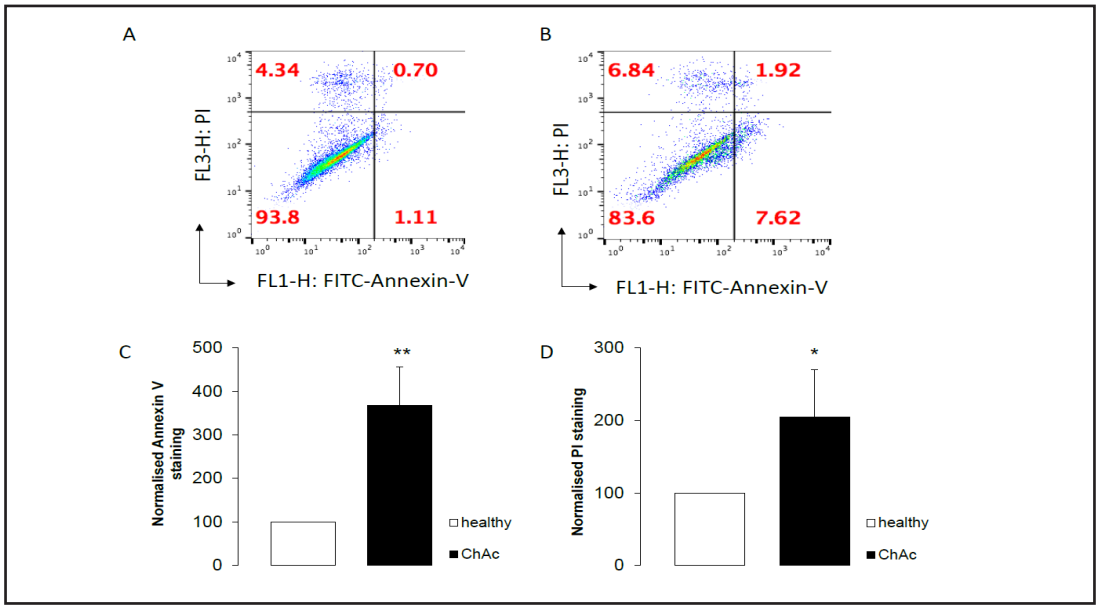

Fig. 4. Phosphatidylserine translocation and propidium iodide uptake in fibroblasts isolated from ChAc patients and healthy volunteers. A,B. Representative dot blots of propidium iodide staining versus annexin-V-binding of fibroblasts isolated from healthy volunteers (A) and from chorea-acanthocytosis (ChAc) patients (B). C. Arithmetic means (± SEM, n = 4-5 individuals) of the percentage of annexin-V-binding fibroblasts from healthy volunteers (white bars) and from ChAc patients (black bars), both expressed in percent of the value in healthy volunteers. D. Arithmetic means (± SEM, n = 4-5 individuals) of the percentage of propidium iodide staining fibroblasts from healthy volunteers (white bars) and from ChAc patients (black bars), both expressed in percent of the value in healthy volunteers. *($p<0.01$) **($p<0.01$) indicates statistically significant difference from healthy individuals (two-tailed unpaired *t*-test).

in untreated ChAc fibroblasts (Fig. 3). The addition of extracellular Ca^{2+} in the continued presence of thapsigargin was followed by a rapid increase of Fura2-fluorescence, which was again significantly higher in lithium treated than in untreated ChAc fibroblasts (Fig. 3). Both peak and slope of SOCE in ChAc fibroblasts were significantly decreased by Orai1 blocker 2-APB (50 µM) (Fig. 3).

In order to test whether the differences in Ca^{2+} signalling are paralleled by differences in apoptosis between ChAc fibroblasts and control fibroblasts, phosphatidylserine exposing cells were identified utilizing annexin-V-binding, and cells with permeable cell membrane utilizing propidium iodide. Both were quantified by flow cytometry. As illustrated in Fig. 4, the percentage annexin-V-binding ChAc fibroblasts was significantly higher than the percentage annexin-V-binding control fibroblasts. Moreover, the percentage of propidium iodide stained fibroblasts was significantly higher in ChAc cells than in control fibroblasts.

Again, additional experiments explored whether phosphatidylserine translocation could be decreased by lithium treatment (2 mM, 24 hours). As shown in Fig. 5, lithium treatment markedly decreased the annexin-V-binding and propidium iodide staining in ChAc fibroblasts. Treatment of the fibroblasts with Orai1 blocker 2-APB (50 µM) was followed by a significant increase of both, annexin-V-binding and propidium iodide staining. In the presence of 2-APB lithium was without significant effect on annexin-V-binding and propidium iodide staining in ChAc fibroblasts (Fig. 5).

Discussion

The present study reveals a novel pathophysiological element in chorea-acanthocytosis, i.e. deranged regulation of store operated Ca^{2+} entry (SOCE). SOCE is significantly down-regulated

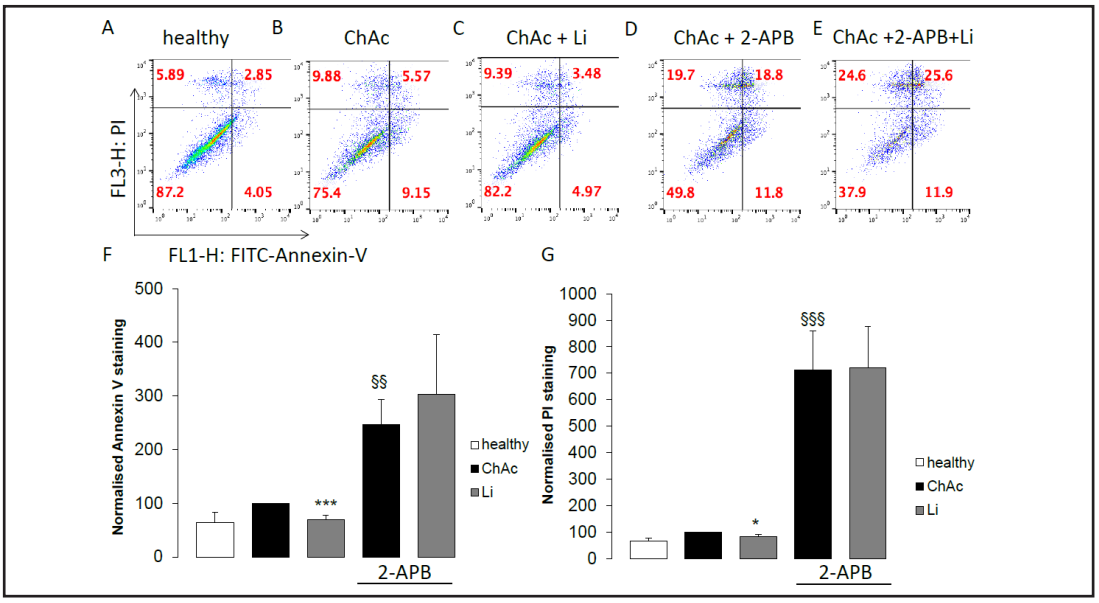

Fig. 5. Phosphatidylserine translocation and propidium iodide uptake in fibroblasts from healthy volunteers and from ChAc patients without and with treatment with lithium and/or 2-APB. A-E. Representative dot blots of propidium iodide staining versus annexin-V-binding of fibroblasts isolated from healthy volunteers (A) and from chorea-acanthocytosis (ChAc) patients (B-E) without treatment (B) and with treatment with lithium (2 mM) alone (C) with 50 µM 2-APB alone (D) and with lithium and 2-APB (E). F. Arithmetic means (± SEM, n = 4-5 individuals) of the percentage annexin-V-binding fibroblasts from healthy volunteers (white bars) and from ChAc patients without (black bars) or with (grey bars) 2 mM lithium treatment without (left bars) and with (right bars) presence of 50 µM 2-APB (2-APB). Values are expressed in percent of the value in untreated ChAc fibroblasts. G. Arithmetic means (± SEM, n = 4-5 individuals) of the percentage propidium iodide staining of fibroblasts from healthy volunteers (white bars) and from ChAc patients without (black bars) or with (grey bars) 2 mM lithium treatment without (left bars) and with (right bars) presence of 50 µM 2-APB (2-APB). Values are expressed in percent of the value in untreated ChAc fibroblasts. *($p<0.05$), ***($p<0.001$) indicates statistically significant difference from untreated ChAc cells, §§($p<0.01$), §§§($p<0.01$) indicates statistically significant difference to respective value in absence of 2-APB, (ANOVA).

in fibroblasts lacking functional chorein. Inhibition of Orai1 with 2-APB triggers suicidal death of fibroblasts from chorea-acanthocytosis (ChAc) patients. SOCE thus apparently confers survival of the fibroblasts. Up-regulation of SOCE favors oscillations of cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$) [44]. The oscillations of $[Ca^{2+}]_i$ result from rapid Ca^{2+} entry due to activation of SOCE followed by similarly rapid Ca^{2+} extrusion. The repetitive short pulses of $[Ca^{2+}]_i$ increase activate Ca^{2+} dependent transcription factors and reorganize the actin filament network without triggering the detrimental consequences of sustained increases of $[Ca^{2+}]_i$ [45]. The Ca^{2+} oscillations participate in the regulation of diverse cellular functions [46-50] including entry into the S and the M phase of the cell cycle [51, 52] and cell survival [53, 54]. In contrast to Ca^{2+} oscillations, sustained increases of cytosolic Ca^{2+} activity trigger apoptosis [48, 55-63]. Along those lines, Orai1, 2, or 3 [19-22], and their regulators STIM 1 or 2 [25, 26, 28] are part of the machinery regulating survival, proliferation, and migration of tumor cells [64-77].

The present study did not address the signalling linking chorein and fibroblast survival. The signalling may well involve PI3K which confers survival of a wide variety of cells including cancer cells [78-92] and neurons [93-96]. PI3K dependent signalling includes the kinases Akt and SGK1 [97], which are both known to up-regulate Orai1 [98, 99].

The present observations further uncover an anti-apoptotic effect of lithium in fibroblasts from chorea-acanthocytosis patients. The effect of lithium is at least in part due to up-regulation of SOCE. Lithium has previously been shown to counteract neurodegeneration [35-42]. In view of the present observations, it is tempting to speculate that lithium may

modify the course of neurodegeneration in part by up-regulating neuronal Orai1 expression and SOCE. As a matter of fact, similar observations on Orai1 expression and SOCE have been made in neurons generated from fibroblasts of ChAc patients [100].

In conclusion, the pathophysiology of chorea-acanthocytosis includes downregulation of the store operated Ca^{2+} entry leading to compromised cell survival. Lithium up-regulates store operated Ca^{2+} entry and attenuates apoptosis.

Acknowledgements

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Disclosure Statement

None.

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