Original Paper

Decrease of Store-Operated Ca^{2+} Entry and Increase of $\text{Na}^{+}/\text{Ca}^{2+}$ Exchange by Pharmacological JAK2 Inhibition

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Key Words

Ca^{2+} release-activated Ca^{2+} channel • SOCE • Orai1 • NCX1 • Janus kinase

Abstract

Background/Aims: Cell proliferation and migration are regulated by cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$). Mechanisms modifying $[\text{Ca}^{2+}]_i$ include store-operated Ca^{2+} -entry (SOCE) accomplished by the pore-forming ion channel unit Orai1 and its regulator STIM1, as well as Ca^{2+} extrusion by $\text{Na}^{+}/\text{Ca}^{2+}$ exchanger NCX1. Kinases participating in the orchestration of cell proliferation include the Janus activated kinase JAK2. The present study explored the impact of pharmacological JAK2 inhibition on SOCE and $\text{Na}^{+}/\text{Ca}^{2+}$ exchange. **Methods:** MCF-7 breast carcinoma cells were studied in the absence and presence of the JAK2 inhibitors TG101348 (250 nM - 1 μM) or of AG490 (20 - 40 μM). Transcript levels were quantified with RT-PCR, protein abundance with immunoblotting, $[\text{Ca}^{2+}]_i$ with Fura-2-fluorescence, SOCE from increase of $[\text{Ca}^{2+}]_i$ following Ca^{2+} re-addition after Ca^{2+} -store depletion with sarcoendoplasmatic Ca^{2+} -ATPase (SERCA) inhibitor thapsigargin (1 μM), and $\text{Na}^{+}/\text{Ca}^{2+}$ exchanger activity from increase of $[\text{Ca}^{2+}]_i$ as well as Ca^{2+} current in whole cell patch clamp following extracellular Na^{+} removal. Migratory activity was determined by a wound healing assay. **Results:** JAK2 inhibitor TG101348 (1 μM) decreased Orai1 and STIM1 protein abundance, increased NCX1 transcript levels, decreased Ca^{2+} release from intracellular stores, decreased SOCE, increased Ca^{2+} entry as well as Ca^{2+} -current following extracellular Na^{+} -removal, and decreased migration. Similar effects on Ca^{2+} release, SOCE, and Ca^{2+} -entry following extracellular Na^{+} -removal were observed following treatment with AG490. **Conclusion:** The present observations disclose a novel powerful mechanism regulating intracellular Ca^{2+} release, cellular Ca^{2+} entry, cellular Ca^{2+} extrusion and cell migration.

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Introduction

Cytosolic Ca^{2+} activity contributes to the orchestration of diverse cellular functions including excitation, exocytosis, migration, cell proliferation and cell death [1-5]. Cytosolic Ca^{2+} concentration is regulated by several mechanisms including Ca^{2+} release from intracellular stores and subsequent activation of store-operated Ca^{2+} entry (SOCE) or Ca^{2+} release-activated Ca^{2+} channel I_{CRAC} , which are accomplished by the pore-forming Ca^{2+} channel subunits Orai1, Orai2 and/or Orai3 [6-10] as well as their regulators STIM1 and/or STIM2 [11-15]. Mechanisms terminating Ca^{2+} signals by Ca^{2+} extrusion include $\text{Na}^{+}/\text{Ca}^{2+}$ -exchange [16-19] by K^{+} -dependent (NCKX) and K^{+} -independent (NCX) $\text{Na}^{+}/\text{Ca}^{2+}$ exchangers [20-24]. Altered Ca^{2+} signaling may well contribute to therapy resistance of tumor cells [25].

A candidate kinase involved in the regulation of Ca^{2+} signaling is janus-activated kinase-2 (JAK2). The kinase contributes to the signaling stimulating cell proliferation and the gain-of-function mutation $\text{V}^{617\text{F}}$ JAK2 is associated with myeloproliferative disease [26-29]. JAK2 inhibitors are therefore considered for the treatment of certain hematological malignancies [30-35]. JAK2 participates in the signaling of a variety of hormones and cytokines [36-38], including leptin [39, 40], growth hormone [41-43], erythropoietin [38], thrombopoietin [38], granulocyte colony-stimulating factor [38] and interferons [44]. Moreover, JAK2 is involved in the cellular response to hypoxia [45]. TG101348 is a selective and potent inhibitor of JAK2 shown to be effective in the treatment of myelofibrosis [46].

The present study explored whether pharmacological inhibition of JAK2 by TG101348 influences Ca^{2+} signaling and migration of MCF-7 breast carcinoma cells.

Materials and Methods

Cell culture

Experiments were performed in MCF-7 breast carcinoma cells cultured in DMEM containing 10% fetal calf serum and 1% antibiotic/antimycotic solution. Where indicated the cells were treated with Orai1 inhibitor YM58483 (Sigma Aldrich, Taufkirchen, Germany) or JAK2 inhibitors TG101348 (Axon Medchem, The Netherlands) [47] and AG490 (Calbiochem, Bad Soden, Germany) [48]. As shown for TG101348, the kinase inhibitors are effective by targeting the adenosine triphosphate (ATP) binding site at the tyrosine kinase domain [49].

Real-time PCR

Total RNA was extracted from MCF-7 breast carcinoma cells in TriFast (Peqlab, Erlangen, Germany) according to the manufacturer's instructions [50]. After DNase digestion reverse transcription of total RNA was performed using Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostics, Penzberg, Germany). Real-time polymerase chain reaction (RT-PCR) of the respective genes were set up in a total volume of 20 μl using 40 ng of cDNA, 500 nM forward and reverse primer and 2x GoTaq® qPCR Master Mix (Promega, Mannheim, Germany) according to the manufacturer's protocol. Cycling conditions were as follows: initial denaturation at 95°C for 2 min, followed by 40 cycles of 95°C for 15 sec, 58°C for 15 sec and 68°C for 20 sec. For amplification the following primers were used (5'→3' orientation):

for Orai1: fw: CGTCCACAACCTCAACTCC; rev: AACTGTCCGGTCCGTCTTAT;

for NCX1: fw: CTGGAATTTCGAGCTCTCCAC; rev: GGCATCATGGAGGTGAAAGT;

for Tbp: fw: ACTCCTGCCACACCAGCC; rev: GGTCAAGTTTACAGCCAAGATTCA

Specificity of PCR products was confirmed by analysis of a melting curve. Real-time PCR amplifications were performed on a CFX96 Real-Time System (Bio-Rad, Munich, Germany) and all experiments were done in duplicate. The house-keeping gene Tbp (TATA binding protein) was amplified to standardize the amount of sample RNA. Relative quantification of gene expression was achieved using the ΔCT method as described earlier [50, 51].

Western blotting

To examine protein abundance, cells were lysed with lysis buffer (Cell Signaling, Frankfurt, Germany) containing protease inhibitor cocktail (Roche) and centrifuged (15,000 g for 20 min at 4°C). Roti Load

buffer (Carl Roth, Karlsruhe, Germany) was added to the protein lysate, which was then incubated at 95°C for 5 min. Proteins were separated by electrophoresis on 10% SDS-PAGE. For immunoblotting, proteins were electro-transferred onto nitrocellulose membranes and blocked with 10% nonfat milk in Tris-buffered saline with 0.1% Tween 20 (TBS-T) at room temperature for 1 h. Then, the membrane was incubated with rabbit anti-Orai1, monoclonal rabbit anti-GAPDH or polyclonal rabbit anti-STIM1 at 4°C overnight. All antibodies were purchased from Cell Signaling and diluted 1:1000 in TBS-T with 5% BSA. After washing with TBST the blots were incubated with secondary goat anti-rabbit antibody (1:3000, Cell Signaling) for 1 h at room temperature. After washing antibody binding was detected with the ECL solution (Amersham ECL Western Blotting Detection Reagents, GE Healthcare, UK). Densitometer scans of the blots were performed using Quantity One (BioRad) [52].

Ca²⁺ measurements

Fura-2 fluorescence was utilized to determine intracellular Ca²⁺ concentrations [53]. 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 phase-contrast 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 Ca²⁺ activity was estimated from the 340 nm/380 nm ratio. SOCE was determined by extracellular Ca²⁺ removal and subsequent Ca²⁺ re-addition in the presence of thapsigargin (1 μM, Invitrogen) [54]. For quantification of Ca²⁺ entry, the slope (delta ratio/s) and peak (delta ratio) were calculated following re-addition of Ca²⁺ [53, 55].

Experiments were performed with Ringer solution containing (in mM): 125 NaCl, 5 KCl, 1.2 MgSO₄, 2 CaCl₂, 2 Na₂HPO₄, 32 HEPES, 5 glucose, pH 7.4. To reach nominally Ca²⁺-free conditions, experiments were performed using Ca²⁺-free Ringer solution containing (in mM): 125 NaCl, 5 KCl, 1.2 MgSO₄, 2 Na₂HPO₄, 32 HEPES, 0.5 EGTA, 5 glucose, pH 7.4.

The changes in [Ca²⁺]_i upon removal of extracellular Na⁺ were taken as measure of Na⁺/Ca²⁺ exchange. N-methyl-D-glucamine (NMDG) was used to replace Na⁺. The Na⁺-standard and Na⁺-free solution contained either 5 mM or 40 mM KCl. Experiments were performed with Ringer solution containing (in mM): 125 NaCl, 5 KCl, 1.2 MgSO₄, 2 CaCl₂, 2 Na₂HPO₄, 32 HEPES, 5 glucose, pH 7.4. To measure Na⁺/Ca²⁺ exchange the standard solution contained (in mM): 130 or 90 NaCl, 5 or 40 KCl, 2 CaCl₂, 2 MgCl₂, 10 HEPES, 5 glucose, pH 7.4, and the Na⁺-free solution contained (in mM): 130 or 90 NMDG, 5 or 40 KCl, 2 CaCl₂, 2 MgCl₂, 10 HEPES, 5 glucose, pH 7.4. For calibration purposes ionomycin (10 μM, Sigma-Aldrich) was applied at the end of each experiment.

Patch clamp

Patch clamp experiments were performed at room temperature in voltage-clamp, fast-whole-cell mode according to Hamill et al. [56]. The cells were continuously superfused through a flow system inserted into the dish. Borosilicate glass pipettes (2–5 MΩ tip resistance; Harvard Apparatus, Kent, UK) manufactured by a microprocessor-driven DMZ puller (Zeitz, Augsburg, Germany) were used in combination with a MS314 electrical micromanipulator (MW, Märzhäuser, Wetzlar, Germany). The currents were recorded by an EPC-9 amplifier (Heka, Lambrecht, Germany) using Pulse software (Heka) and an ITC-16 Interface (Instrutech, Port Washington, N.Y., USA) [57, 58]. The currents were recorded with an acquisition frequency of 10 kHz and 3 kHz low-pass filtered. The offset potentials between both electrodes were zeroed before sealing. Whole-cell currents elicited by changing the bath solutions were measured during a continuous 40-second square wave voltage pulse to -80 mV. The applied voltages refer to the cytoplasmic face of the membrane with respect to the extracellular space. The outward currents, defined as flow of positive charge from the cytoplasmic to the extracellular membrane face, are positive currents and depicted as upward deflections of the original current traces. To measure Na⁺/Ca²⁺ exchanger-mediated currents, a Na⁺-based pipette solution was used (in mM): 120 NaCl, 40 KCl, 20 TEA-Cl, 2 MgCl₂, 2 Mg-ATP, 10 HEPES (pH 7.2/CsOH) and 1 μM free Ca²⁺. The external first solution contained (in mM): 130 NaCl, 20 TEA-Cl, 2 MgCl₂, 10 glucose, 10 HEPES, 0.5 EGTA (pH 7.2/CsOH). Na⁺/Ca²⁺ exchange currents were elicited by switching from the first bath solution to a bath solution that contained (in mM): 130 NMDG-Cl, 20 TEA-Cl, 2 MgCl₂, 2 CaCl₂, 10 glucose and 10 HEPES (pH 7.2/CsOH).

Wound healing Assay

Cell migration was measured using a wound healing assay [59]. Cells were seeded in 6-well plates at a density of 2×10^5 cells/mL and grown to confluence. The monolayer of cells was scratched with a 200 μ L pipette tip to create a wound gap, and treated with either TG101348 (1 μ M, 24h) or YM58483 (1 μ M). Cell images were obtained with a digital camera connected to a phase-contrast Olympus microscope. The same visual field was used throughout the experiment. The wound gap was measured by ImageJ software. Wound closure was calculated using the following formula: Wound closure (%) = $\text{Gap}(T-T_0)/\text{Gap}T_0 \times 100\%$ (where T is the treatment time and T0 is the time that the wound was induced)

Statistical analysis

Data are provided as means $\pm$ SEM; *n* represents the number of independent experiments. All data were tested for significance using Student's unpaired two-tailed *t*-test, one sample *t*-test or ANOVA (Dunnett's test), where applicable. Results with $p < 0.05$ were considered statistically significant.

Results

Inhibition of Orai1, STIM1, intracellular Ca^{2+} release and SOCE by JAK2 inhibitors TG101348 and AG490

RT-PCR was employed to explore whether MCF-7 carcinoma cells transcribe Orai1 and whether the Orai1 transcript level is sensitive to the JAK2 inhibitor TG101348. As shown in Fig. 1, Orai1 was indeed transcribed in MCF-7 breast carcinoma cells. A 24 hours treatment with TG101348 (1 μ M) significantly decreased the Orai1 transcript levels.

Orai1 and STIM1 protein abundance was determined by Western blots. As shown in Fig. 2, Orai1 and STIM1 were expressed in MCF-7 cells. A 24 hours treatment with TG101348 (1 μ M) significantly decreased the Orai1 and STIM1 protein levels.

Fura2-fluorescence was taken as measure of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$). In order to test whether the differences in Orai1 transcript levels were paralleled by corresponding differences in store-operated Ca^{2+} entry (SOCE), intracellular 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 μ M) followed by re-addition of extracellular Ca^{2+} in the continued presence of thapsigargin. The Fura2-fluorescence ratio prior to extracellular Ca^{2+} removal was similar in untreated (0.38 ± 0.01 a.u., $n = 47$) and TG101348-treated (1 μ M, 24 h) (0.39 ± 0.01 a.u., $n = 59$) MCF-7 cells. As illustrated in Fig. 3, the addition of thapsigargin triggered Ca^{2+} release from intracellular stores, leading to rapid, transient increase in cytosolic Ca^{2+} activity. The increase of intracellular Ca^{2+} activity following thapsigargin treatment was significantly smaller in TG101348 treated than in untreated MCF-7 carcinoma cells (Fig. 3). The increase of intracellular Ca^{2+} activity following thapsigargin treatment was further significantly smaller in MCF-7 cells treated with AG490, another JAK2 inhibitor, compared to untreated cells (Fig. 4). The addition of extracellular Ca^{2+} in the continued presence of thapsigargin was followed by a rapid increase of Fura2-fluorescence

Fig. 1. Transcript levels of Orai1 in MCF-7 cells without and with TG101348 treatment. Arithmetic means $\pm$ SEM ($n = 12$) of Orai1 transcript levels in untreated (white bars) and TG101348 (1 μ M, 24 h) treated (black bars) MCF-7 breast carcinoma cells. *** ($p < 0.001$) indicates statistically significant difference from untreated cells (two-tailed unpaired *t*-test).

Fig. 2. Orai1 and STIM1 protein abundance in MCF-7 cells without and with TG101348 treatment. A. Original Western blot showing the protein abundance of Orai1 and GAPDH in MCF-7 cells without (control) and with (TG101348) prior TG101348 (1 μ M, 24 h) treatment. B. Arithmetic means $\pm$ SEM (n = 3 independent experiments) of Orai1 over GAPDH protein abundance in untreated (white bars) and TG101348 (1 μ M, 24 h) treated (black bars) MCF-7 cells. C. Original Western blot showing the protein abundance of STIM1 and GAPDH in MCF-7 cells without (control) and with (TG101348) prior TG101348 (1 μ M, 24 h) treatment. D. Arithmetic means $\pm$ SEM (n = 5 independent experiments) of STIM1 over GAPDH protein abundance in untreated (white bars) and TG101348 (1 μ M, 24 h) treated (black bars) MCF-7 cells. * (p<0.05), ** (p<0.01) indicates significant difference from control (unpaired t-test).

Fig. 3. Intracellular Ca²⁺ release and store-operated Ca²⁺ entry (SOCE) in MCF-7 cells without and with TG101348 treatment. A. Representative tracings of Fura-2 fluorescence-ratio in fluorescence spectrometry before and following extracellular Ca²⁺ removal and addition of thapsigargin (1 μ M), as well as re-addition of extracellular Ca²⁺ in untreated (white symbols) and TG101348 (250 nM, 500 nM, 1 μ M, 24 h) treated (light grey, dark grey and black symbols) MCF-7 carcinoma cells. B,C. Arithmetic means ($\pm$ SEM, n = 37 - 73) of slope (B) and peak (C) increase of fura-2-fluorescence-ratio following addition of thapsigargin (1 μ M) in untreated (white bars) and TG101348 (250 nM, 500 nM, 1 μ M, 24 h) treated (light grey, dark grey and black bars) MCF-7 carcinoma cells. D,E. Arithmetic means ($\pm$ SEM, n = 37 - 73) of slope (D) and peak (E) increase of fura-2-fluorescence-ratio following re-addition of extracellular Ca²⁺ in untreated (white bars) and TG101348 (250 nM, 500 nM, and 1 μ M, 24 h) treated (light grey, dark grey and black bars) MCF-7 carcinoma cells. * (p<0.05), ** (p<0.01), *** (p<0.001) indicates statistically significant difference from untreated cells (two-tailed unpaired t-test).

reflecting store operated Ca²⁺ entry (SOCE) in both, treated and untreated cells. Both, peak and slope of SOCE were significantly lower in TG101348-treated (Fig. 3) and AG490-treated (Fig. 4) than in untreated MCF-7 carcinoma cells.

Fig. 4. Intracellular Ca^{2+} release and store-operated Ca^{2+} entry (SOCE) in MCF-7 cells without and with AG490 treatment. A. Representative tracings of Fura-2 fluorescence-ratio in fluorescence spectrometry before and following extracellular Ca^{2+} removal and addition of thapsigargin ($1 \mu\text{M}$, 24 h), as well as re-addition of extracellular Ca^{2+} in untreated (white symbols) and AG490 (20 – 40 μM , 24 h) treated (grey, black symbols) MCF-7 carcinoma cells. B,C. Arithmetic means ($\pm$ SEM, $n = 43 - 57$) of slope (B) and peak (C) increase of fura-2-fluorescence-ratio following addition of thapsigargin ($1 \mu\text{M}$) in untreated (white bars) and AG490 (20 - 40 μM , 24 h) treated (grey, black bars) MCF-7 carcinoma cells. D,E. Arithmetic means ($\pm$ SEM, $n = 43 - 57$) of slope (D) and peak (E) increase of fura-2-fluorescence-ratio following re-addition of extracellular Ca^{2+} in untreated (white bars) and AG490 (20 - 40 μM , 24 h) treated (grey, black bars) MCF-7 carcinoma cells. **($p < 0.01$), *** ($p < 0.001$) indicates statistically significant difference from untreated cells (two-tailed unpaired t -test).

Fig. 5. ATP-induced Ca^{2+} release in MCF-7 cells without and with TG101348 treatment. A. Representative tracings showing the Fura-2 fluorescence ratio reflecting cytosolic Ca^{2+} activity in untreated (white symbols) and TG101348 ($1 \mu\text{M}$, 24 h) treated (black symbols) MCF-7 breast carcinoma cells. Ca^{2+} was removed from and ATP ($100 \mu\text{M}$) added to the bath solution leading to release of Ca^{2+} from intracellular stores with subsequent increase of the Fura-2 fluorescence ratio. The amplitude (peak) and the velocity (slope, calculated from the linear fit) of the Fura-2 fluorescence ratio increase were analyzed. B,C. Arithmetic means ($\pm$ SEM, $n = 58 - 60$ cells) of the slope (B) and the peak (C) of the change in Fura-2 fluorescence ratio in MCF-7 cells without (white bars) and with TG101348 ($1 \mu\text{M}$, 24 h) treatment (black bars) following addition of ATP ($100 \mu\text{M}$) in the absence of Ca^{2+} (Ca^{2+} release). ***($p < 0.001$) indicates significant difference from untreated cells (two-tailed unpaired t -test).

In order to define the impact of JAK2 on agonist-induced Ca^{2+} release from intracellular stores, the effect of purinergic receptor agonist ATP in the absence of extracellular Ca^{2+} was tested. As illustrated in Fig. 5, addition of ATP ($100 \mu\text{M}$) in the absence of extracellular Ca^{2+} was followed by transient increase of Fura-2 fluorescence ratio reflecting Ca^{2+} release from intracellular stores. Amplitude (peak, Δ ratio) and velocity (slope, Δ ratio/s) were both significantly less pronounced in TG101348 ($1 \mu\text{M}$, 24 h) treated than untreated MCF-7 cells (Fig. 5). Accordingly, lack of JAK2 compromised ATP-induced intracellular Ca^{2+} release.

Fig. 6. Transcript levels of NCX1 in MCF-7 cells without and with TG101348 treatment. Arithmetic means $\pm$ SEM (n = 13) of NCX1 transcript levels in untreated (white bars) and TG101348 (1 μ M, 24 h) treated (black bars) MCF-7 breast carcinoma cells. * (p<0.05) indicates statistically significant difference from untreated cells (two-tailed unpaired t-test).

Fig. 7. NCX-mediated Ca^{2+} entry in MCF-7 cells without and with TG101348 treatment. A. Representative original tracings showing intracellular Ca^{2+} concentrations in Fura-2/AM loaded untreated (white symbols) and TG101348 (250 nM, 500 nM, 1 μ M, 24 h) treated (light grey, dark grey, black symbols) MCF-7 breast carcinoma cells prior to and following removal of external Na^+ (0 Na^+). The amplitude (peak) and the velocity (slope, calculated from the linear fit) of the Ca^{2+} entry were analyzed. B,C. Mean ($\pm$ SEM, n = 17 - 49 cells) of the peak value (B) and slope (C) of the change in intracellular Ca^{2+} concentrations in untreated (white bars) and TG101348 (250 nM, 500 nM, 1 μ M, 24 h) treated (light grey, dark grey, black bars) MCF-7 breast carcinoma cells following removal of external Na^+ . *** (p<0.001) indicate significant differences from untreated cells (two-tailed unpaired t-test).

Increase of NCX by JAK2 inhibitors TG101348 and AG490

Ca^{2+} extrusion involves the $\text{Na}^+/\text{Ca}^{2+}$ exchanger NCX. RT-PCR was employed to test whether MCF-7 breast carcinoma cells express NCX1 and whether JAK2 inhibitor TG101348 influences the transcript levels of NCX1. As shown in Fig. 6, NCX1 was transcribed in MCF-7 breast carcinoma cells. A 24 h treatment with TG101348 (1 μ M) significantly increased the NCX1 transcript levels.

Again, Fura2-fluorescence was employed in order to test whether the differences in NCX1 transcript levels were paralleled by corresponding differences in $\text{Na}^+/\text{Ca}^{2+}$ exchanger activity. The changes in cytosolic Ca^{2+} activity upon removal of extracellular Na^+ were taken as measure of $\text{Na}^+/\text{Ca}^{2+}$ exchange. As illustrated in Fig. 7, removal of extracellular Na^+ was followed by an increase of cytosolic Ca^{2+} activity in treated and untreated MCF-7 cells. The effect was, however, significantly larger in TG101348 treated cells than in untreated cells. Moreover, the effect was significantly larger in AG490-treated cells than in untreated cells (Fig. 8).

$\text{Na}^+/\text{Ca}^{2+}$ exchanger activity was further quantified by measurements of whole cell currents utilizing the patch clamp technique. As illustrated in Fig. 9, the whole cell currents following a switch between external solutions from 130 mM Na^+ and no Ca^{2+} (130 Na^+ 0 Ca^{2+}) to 2 mM Ca^{2+} and no Na^+ (0 Na^+ 2 Ca^{2+}) was significantly larger in TG101348 (1 μ M, 24 h)

Fig. 8. NCX-mediated Ca^{2+} entry in MCF-7 cells without and with AG490 treatment. A. Representative original tracings showing intracellular Ca^{2+} concentrations in Fura-2/AM loaded untreated (white symbols) and AG490 (20 - 40 μM , 24 h) treated (grey, black symbols) MCF-7 breast carcinoma cells prior to and following removal of external Na^+ (0 Na^+). The amplitude (peak) and the velocity (slope, calculated from the linear fit) of the Ca^{2+} entry were analyzed. B,C. Mean ($\pm$ SEM, $n = 37 - 56$ cells) of the peak value (B) and slope (C) of the change in intracellular Ca^{2+} concentrations in untreated (white bars) and AG490 (20 - 40 μM , 24 h) treated (grey, black bars) MCF-7 breast carcinoma cells following removal of external Na^+ . *** ($p < 0.001$) indicate significant differences from untreated cells (two-tailed unpaired t -test).

Fig. 9. NCX $\text{Na}^+/\text{Ca}^{2+}$ exchanger currents in MCF-7 cells without and with TG101348 treatment. A. Original tracings of whole cell currents in untreated (left) and TG101348 (1 μM , 24 h) treated (right) MCF-7 breast carcinoma cells recorded at -80 mV during the switch between external solutions containing either 130 mM Na^+ and no Ca^{2+} (130 Na^+ , 0 Ca^{2+}) or 2 mM Ca^{2+} and no Na^+ (0 Na^+ , 2 Ca^{2+}). The internal solution contained 1 μM free Ca^{2+} , 120 mM Na^+ , 40 mM K^+ . Cesium (2 mM) and TEA $^+$ (20 mM) were added to block K^+ channels. B. Mean current density changes (ΔI , pA/pF) at -80 mV in untreated (white bars) ($n = 6$) and TG101348 (1 μM) treated (black bars) ($n = 8$) MCF-7 breast carcinoma cells induced by the switch between external solutions that contained either 130 mM Na^+ and no Ca^{2+} (130 Na^+ , 0 Ca^{2+}) or 2 mM Ca^{2+} and no Na^+ (0 Na^+ , 2 Ca^{2+}). ** ($p < 0.01$) indicates statistically significant difference from in untreated cells (two-tailed unpaired t -test).

treated than in untreated MCF-7 breast carcinoma cells. The patch clamp experiments thus confirmed up-regulation of NCX-mediated current by TG101348 treatment.

Impaired migration of MCF-7 cells following treatment with TG101348 or Orai1 inhibitor YM58483

A final series of experiments addressed whether TG101348 treatment affected migration, a known Ca^{2+} -sensitive cell function. As illustrated in Fig. 10, MCF-7 breast carcinoma cells rapidly migrated, thus decreasing the percentage of wound area in a wound-healing assay. Fig. 10 further demonstrates that treatment with TG101348 (1 μM , 24 h) significantly

Fig. 10. Migration of MCF-7 cells without and with TG101348 treatment. A. Original photographs illustrating migration of MCF-7 cells without and with a 24 hours treatment with TG101348 (1 μ M) in the presence or absence of Orai1 inhibitor YM58483 (100 nM, 24 h) in a wound healing assay at 0 hours (left panels) and 24 hours (right panels) after wounding. B. Arithmetic

means $\pm$ SEM (n = 3) of the percentage of wound closure determined by image analysis without (white bar) and with a 24 hours treatment with (black bar) TG101348 (1 μ M, 24 h) in the presence (light grey bar) or absence (dark grey bar) of Orai1 inhibitor YM58483 (100 nM). * (p < 0.05), *** (P < 0.001) indicates statistically significant difference from untreated cells, ANOVA.

delayed the decrease of wound area. The migration of MCF-7 cells was virtually abolished by Orai1 inhibitor YM58483 (100 nM) and the combined treatment with TG101348 and YM58483.

Discussion

The present study suggests that Janus-activated kinase JAK2 is a completely novel regulator of Ca^{2+} signaling in MCF-7 breast carcinoma cells. As shown in those cells, pharmacological inhibition of JAK2 by TG101348 decreases both Orai1 and STIM1 protein abundance, Ca^{2+} release from intracellular stores and store operated Ca^{2+} entry (SOCE). Moreover, TG101348 increases NCX transcript levels and $\text{Na}^{+}/\text{Ca}^{2+}$ exchanger activity in MCF-7 breast carcinoma cells. The study further demonstrates that TG101348 interferes with migration of MCF-7 breast carcinoma cells. To the extent that treatment with TG101348 is effective by inhibiting JAK2, the data suggest that JAK2 increases Ca^{2+} release from intracellular stores, up-regulates store operated Ca^{2+} entry (SOCE) and decreases Ca^{2+} extrusion by $\text{Na}^{+}/\text{Ca}^{2+}$ exchange.

Up-regulation of SOCE has been shown to trigger Ca^{2+} oscillations [60], which are further augmented by compromised $\text{Na}^{+}/\text{Ca}^{2+}$ exchange [60]. The Ca^{2+} oscillations stimulate several cellular functions [17, 61-64] including entry into the S and the M phase of the cell cycle [65, 66] and cell survival [67, 68]. In contrast, sustained increase of cytosolic Ca^{2+} activity leads to apoptosis [62, 64, 69-77].

Previous observations documented the role of Ca^{2+} channel units Orai1, 2, or 3 [6-9] and their regulators STIM 1 or 2 [12, 13, 15] in survival, proliferation, and migration of tumor cells [39, 78-90]. Augmented Orai1 expression and Ca^{2+} entry may thus contribute to the known stimulating effect of JAK2 on cell proliferation [26-29] and, as shown in this study, on cell migration.

In conclusion, the present study provides pharmacological evidence suggesting that JAK2 up-regulates Ca^{2+} release from intracellular stores, up-regulates store operated Ca^{2+}

entry (SOCE) and decreases Ca^{2+} extrusion by $\text{Na}^{+}/\text{Ca}^{2+}$ exchange, thus fostering Ca^{2+} sensitive functions, such as cell migration.

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Disclosure Statement

The authors of this manuscript declare that they have no conflicts of interests

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