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Downregulation of Chloride Channel ClC-2 by Janus Kinase 3

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Abstract Janus kinase-3 (JAK3) fosters proliferation and counteracts apoptosis of lymphocytes and tumor cells. The gain of function mutation ^{A572V}JAK3 has been discovered in acute megakaryoplastic leukemia. JAK3 is inactivated by replacement of lysine by alanine in the catalytic subunit (^{K855A}JAK3). Regulation of cell proliferation and apoptosis involves altered activity of Cl^- channels. The present study, thus, explored whether JAK3 modifies the function of the small conductance Cl^- channel ClC-2. To this end, ClC-2 was expressed in *Xenopus* oocytes with or without wild-type JAK3, ^{A568V}JAK3 or ^{K851A}JAK3, and the Cl^- channel activity determined by dual-electrode voltage clamp. Channel protein abundance in the cell membrane was determined utilizing chemiluminescence. As a result, expression of ClC-2 was followed by a marked increase of cell membrane conductance. The conductance was significantly decreased following coexpression of JAK3 or ^{A568V}JAK3, but not by coexpression of ^{K851A}JAK3. Exposure of the oocytes expressing ClC-2 together with ^{A568V}JAK3 to the JAK3 inhibitor WHI-P154 (4-[(3'-bromo-4'-hydroxyphenyl)amino]-6,7-dimethoxyquinazoline, 22 μM) increased the conductance. Coexpression of ^{A568V}JAK3 decreased the ClC-2 protein abundance in the cell membrane of ClC-2 expressing oocytes. The decline of conductance in ClC-2 and ^{A568V}JAK3 coexpressing oocytes following inhibition of channel protein insertion by brefeldin A (5 μM) was similar in oocytes expressing ClC-2 with ^{A568V}JAK3 and oocytes expressing ClC-2 alone, indicating that ^{A568V}JAK3 might slow channel protein insertion into rather than accelerating channel protein retrieval from the cell membrane. In conclusion, JAK3 downregulates ClC-2 activity and thus counteracts Cl^- exit—an effect possibly influencing cell proliferation and apoptosis.

Keywords Cell proliferation · Apoptosis · Cell volume regulation · Chloride channels

Introduction

Janus kinase 3 (JAK3), a tyrosine kinase contributing to the signaling of hematopoietic cell cytokine receptors (Cornejo et al. 2009; Imada and Leonard 2000; Ghoreschi et al. 2009; O'Shea et al. 2002; Shuai and Liu 2003), fosters cell proliferation and counteracts apoptosis of lymphocytes and tumor cells (de Totero et al. 2008; Fainstein et al. 2008; Nakayama et al. 2009; Kim et al. 2010; Uckun et al. 2007). The gain of function JAK3 mutation ^{A572V}JAK3 has been discovered in acute megakaryoplastic leukemia (Malinge et al. 2008; Walters et al. 2006). JAK3 is inactivated by replacement of the ATP coordinating lysine by alanine in the catalytic subunit (^{K855A}JAK3) (Haan et al. 2011).

Cell proliferation critically depends on the activity of ion channels including Cl^- channels (Lang et al. 2007). Activation of Cl^- channels results in cell shrinkage, which is decisive for the triggering of oscillations of cytosolic Ca^{2+} activity (Ritter et al. 1993). Anion channels involved in cell volume regulatory decrease (Furukawa et al. 1998; Grunder et al. 1992; Thiemann et al. 1992) include the ubiquitously expressed and highly conserved inwardly rectifying Cl^- channel ClC-2 (Jentsch et al. 1995; Thiemann et al. 1992). Cell shrinkage inhibits Cl^- channels, thus decreasing cellular Cl^- loss (Lang et al. 1998; Macri et al. 1997). Cl^- channels are typically activated during

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apoptosis (Elinder et al. 2005; Lang et al. 2006; Myssina et al. 2004; Okada and Maeno 2001; Okada et al. 2004; Porcelli et al. 2004; Shimizu et al. 2004; Souktani et al. 2000; Szabo et al. 1998; Wei et al. 2004; Zuo et al. 2009).

The present study explored, whether JAK3 participates in the regulation of ClC-2. To this end, ClC-2 was expressed in *Xenopus* oocytes with or without wild-type JAK3, active ^{A568V}JAK3 or inactive ^{K851A}JAK3. ClC-2 induced currents were determined utilizing dual-electrode voltage clamp and ClC-2 protein abundance in the cell membrane estimated from chemiluminescence. As a result, coexpression of JAK3 and of active ^{A568V}JAK3 but not coexpression of ^{K851A}JAK3 decreased ClC-2 induced conductance in ClC-2-expressing *Xenopus* oocytes. Furthermore, coexpression of active ^{A568V}JAK3 diminished ClC-2 protein abundance in ClC-2-expressing *Xenopus* oocytes.

Materials and Methods

Constructs

For generation of cRNA, cDNA constructs encoding wild-type human ClC-2 (Pusch et al. 1999; Kowalczuk et al. 2008; Stegen et al. 2000) and rat hemagglutinin (HA)-tagged ClC-2 (Hosseinzadeh et al. 2012b), as well as wild-type mouse JAK3, inactive ^{K851A}JAK3 mutant, and the gain of function ^{A568V}JAK3 mutant (Warsi et al. 2013), were used. The cRNA was generated as described previously (Hosseinzadeh et al. 2013b; Broer et al. 1994).

Voltage Clamp in *Xenopus* oocytes

Xenopus oocytes were prepared as previously described (Munoz et al. 2013; Shojaiefard et al. 2012). The oocytes were injected with 15 ng cRNA encoding wild-type ClC-2 or ClC-2-HA, as well as wild-type JAK3 cRNA or ^{A568V}JAK3 or ^{K851A}JAK3 cRNA (10 ng) on the first day after preparation of the oocytes (Hosseinzadeh et al. 2013a; Almilaji et al. 2013a). The oocytes were maintained at 17°C in ND96 solution containing (in mM): 88.5 NaCl, 2 KCl, 1 $MgC1_2$, 1.8 $CaC1_2$, 2.5 NaOH, 5 HEPES, and 5 Sodium Pyruvate ($C_3H_3NaO_3$) (pH 7.4). The ND96 solution was supplemented with 100 mg/l gentamycin, 50 mg/l Tetracycline, 1.6 mg/l Ciprofloxacin, and 90 mg/l Theophylline; and, where indicated, with JAK3 inhibitor WHI-P154 (4-[(3'-bromo-4'-hydroxyphenyl)amino]-6,7-dimethoxyquinazoline, 22 μM final concentration) or brefeldin A (5 μM final concentration). Experiments were performed at room temperature 3 days after injection. The currents were determined in two-electrode voltage clamp utilizing a pulse protocol of 10 s pulses from −140 to +40 mV in 20 mV increments. The intermediate holding voltage was −60 mV. The current at the end of each voltage step was taken for data analysis (Hosseinzadeh et al. 2012a; Alesutan et al. 2012). The data were filtered at 2 kHz, and recorded with a DigiData 1300 A/D-D/A converter and the pClamp 9.0 software for data acquisition and analysis (Axon Instruments, USA) (Pathare et al. 2012a; Pathare et al. 2012b). The bath solution ND96 was used for the experiments (Mia et al. 2012). The flow rate of the superfusion was 20 ml/min, and a complete exchange of the bath solution was reached within about 10 s (Almilaji et al. 2013b; Bogatikov et al. 2012).

Detection of ClC-2 Cell Surface Expression by Chemiluminescence

To determine ClC-2 cell surface expression by chemiluminescence, defolliculated oocytes were injected with water or 15 ng cRNA encoding ClC-2-HA, which contains an inserted HA epitope (Pakladok et al. 2013), with or without 10 ng cRNA encoding ^{A568V}JAK3. Oocytes were incubated with mouse monoclonal anti-HA antibody conjugated to Horseradish Peroxidase (1:1,000, Miltenyi Biotec Inc, CA, USA) for 1 h. After staining, individual oocytes were placed in 96-well plates with 20 μL of SuperSignal ELISA Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL, USA); and chemiluminescence of single oocytes was quantified in a luminometer (Walter Wallac 2 plate reader, Perkin Elmer, Juegesheim, Germany) by integrating the signal over a period of 1 s. The results of the experiments are given as normalized relative light units (Pakladok et al. 2012).

Statistical Analysis

Data are provided as means ± SEM, n represents the number of oocytes investigated. All experiments were repeated with at least 2–3 batches of oocytes; in all repetitions qualitatively similar data were obtained. Data were tested for significance using ANOVA or *t* test, as appropriate. Results with $p < 0.05$ were considered statistically significant.

Results

The present study explored the effect of Janus kinase 3 (JAK3) on the activity of ClC-2 Cl^- channels. To this end,

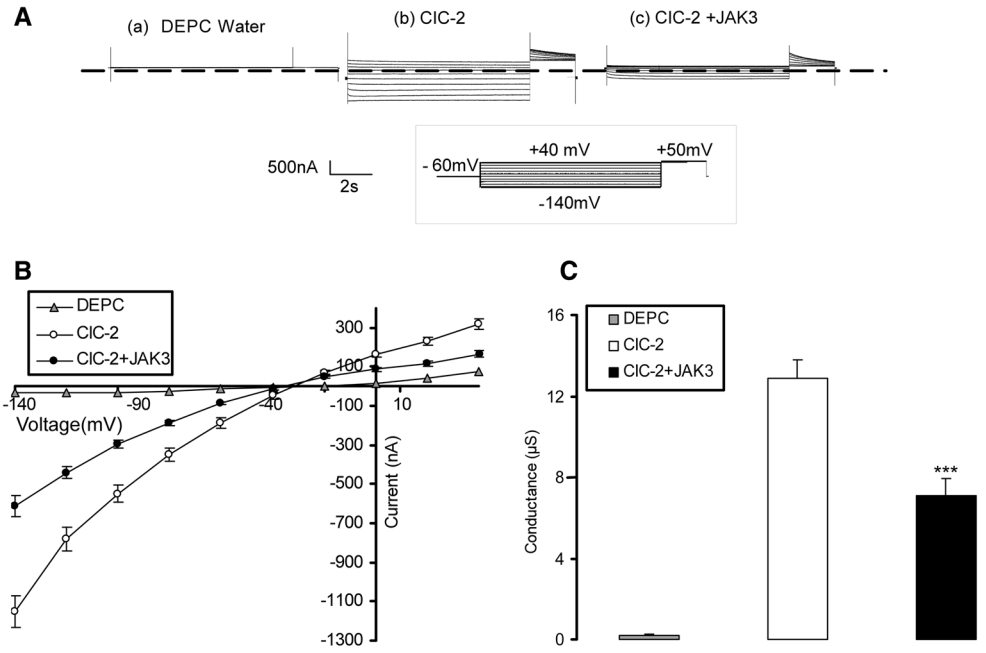

Fig. 1 Coexpression of JAK3 decreases Cl^- conductance in ClC-2-expressing *Xenopus* oocytes **A**: Representative original tracings showing currents in *Xenopus* oocytes injected with DEPC water (a), as well as in oocytes expressing ClC-2 without (b) or with additional coexpression of wild-type JAK3 (c) **B**: Arithmetic means ± SEM ($n = 20$–21) of the current (I) as a function of the potential difference across the cell membrane (V) in *Xenopus* oocytes injected with water (DEPC water, *gray triangles*), expressing ClC-2 alone (ClC-2, white circles) or expressing ClC-2 together with wild-type JAK3 (ClC-2 + JAK3, *black circles*) **C**: Arithmetic means ± SEM ($n = 20$–21) of the conductance calculated by linear fit of I/V curves shown in **B** between −140 and −80 mV in *Xenopus* oocytes injected with water (DEPC water, *dotted bar*), expressing ClC-2 alone (ClC-2, *white bar*) or expressing ClC-2 together with wild-type JAK3 (ClC-2 + JAK3, *black bar*) ***($p < 0.001$) indicates statistically significant difference to expression of ClC-2 alone

cRNA encoding ClC-2 was injected into *Xenopus* oocytes with or without additional injection of cRNA encoding JAK3 and the cell membrane conductance was determined utilizing dual-electrode voltage clamp. In water-injected oocytes, the cell membrane conductance was low (Fig. 1). As shown earlier (Hosseinzadeh et al. 2012b), expression of ClC-2 resulted in a marked increase of cell membrane conductance. As shown in Fig. 1, additional expression of wild-type JAK3 was followed by a significant decrease of the current in ClC-2-expressing oocytes.

The effects of wild-type JAK3 were mimicked by the constitutively active mutant ^{A568V}JAK3. Coexpression of ^{A568V}JAK3 significantly decreased Cl^- channel activity in ClC-2-expressing oocytes. In *Xenopus* oocytes expressing ClC-2 together with ^{A568V}JAK3, the conductance was significantly lower than in *Xenopus* oocytes expressing ClC-2 alone (Fig. 2). In contrast, ClC-2 activity was not significantly modified by the inactive mutant ^{K851A}JAK3 (Fig. 2).

Pharmacological inhibition of JAK3 with JAK3 inhibitor WHI-P154—4-[(3'-bromo-4'-hydroxyphenyl)amino]-6,7-dimethoxyquinazoline (22 µM) reversed the effect of ^{A568V}JAK3. As illustrated in Fig. 3, the Cl^- current in ClC-2- and ^{A568V}JAK3-expressing *Xenopus* oocytes was significantly increased following treatment with the JAK3 inhibitor WHI-P154 (22 µM) (Fig. 3).

The downregulation of ClC-2 activity by JAK3 or ^{A568V}JAK3 may have resulted from an influence of the kinase on the channel protein abundance in the cell membrane. Chemiluminescence was, thus, employed to quantify channel protein abundance in the plasma membrane. As illustrated in Fig. 4, the coexpression of ^{A568V}JAK3 was followed by a significant decrease of ClC-2-HA protein abundance in the *Xenopus* oocytes cell membrane (Fig. 4).

JAK3 or ^{A568V}JAK3 could decrease ClC-2 protein abundance in the cell membrane either by impeding channel protein insertion or by accelerating channel protein retrieval. In order to discriminate between these two possibilities, ClC-2- and ^{A568V}JAK3-expressing *Xenopus* oocytes were treated with 5 µM brefeldin A—a substance disrupting insertion of new channel protein into the cell membrane. As illustrated in Fig. 5, the decline of conductance in the presence of brefeldin A was similar in oocytes expressing ClC-2 together with ^{A568V}JAK3 and oocytes expressing ClC-2 alone.

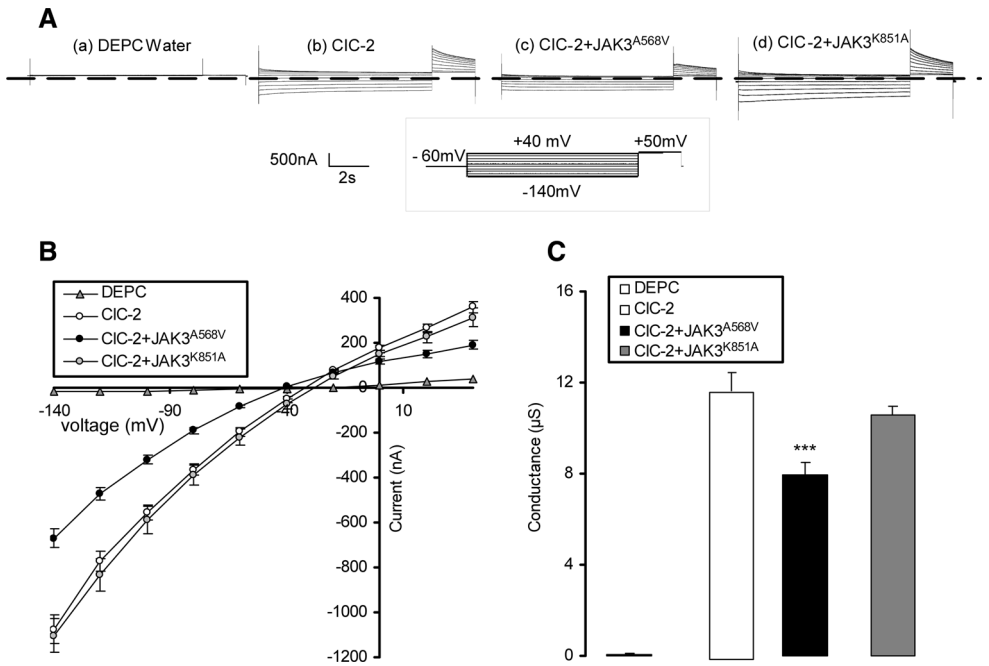

Fig. 2 The effect of wild-type JAK3 is mimicked by active ^{A568V}JAK3, but not by inactive ^{K851A}JAK3 **A**: Representative original tracings showing currents in *Xenopus* oocytes injected with DEPC water (a) or expressing ClC-2 alone (b) or expressing ClC-2 together with constitutively active ^{A568V}JAK3 (c) or inactive ^{K851A}JAK3 (d) **B**: Arithmetic means ± SEM ($n = 17$–44) of the current (I) as a function of the potential difference across the cell membrane (V) in *Xenopus* oocytes injected with water (DEPC water, *gray triangles*), expressing ClC-2 alone (ClC-2, *white circles*) or expressing ClC-2 together with constitutively active ^{A568V}JAK3 (*black circles*) or with inactive ^{K851A}JAK3 (light gray circles) **C**: Arithmetic means ± SEM ($n = 17$–44) of the conductance calculated by linear fit of I/V curves shown in B between −140 and −80 mV in *Xenopus* oocytes injected with water (DEPC water, *white bar*), expressing ClC-2 alone (ClC-2, *white bar*) or expressing ClC-2 together with constitutively active ^{A568V}JAK3 (ClC-2 + ^{A568V}JAK3, black bar) or with inactive ^{K851A}JAK3 (ClC-2 + ^{K851A}JAK3, light *gray bar*). *** ($p < 0.001$) indicates statistically significant difference to expression of ClC-2 alone

Discussion

The present observations reveal a novel mechanism in the regulation of the Cl^- channel ClC-2. Janus kinase 3 decreases the ClC-2 protein abundance in the cell membrane and thus Cl^- channel activity. The experiments with brefeldin A are suggestive for an effect of JAK3 on channel protein insertion into rather than channel protein retrieval from the cell membrane.

The effect of JAK3 on ClC-2 may impact on cell volume, as inhibition of Cl^- channels curtails Cl^- exit—thus fostering hyperpolarization of the cell membrane and decreasing K^+ exit with osmotically obliged water. Cell shrinkage leads to inhibition of cell volume regulatory Cl^- channels (Lang et al. 1998; Macri et al. 1997). ClC-2 has been shown to be sensitive to cell volume (Grunder et al. 1992). At least in theory, JAK3 may participate in the regulation of the channel following cell shrinkage.

Activation of Cl^- channels is further involved in the regulation of apoptosis (Elinder et al. 2005; Lang et al. 2006; Myssina et al. 2004; Okada and Maeno 2001; Okada et al. 2004; Porcelli et al. 2004; Shimizu et al. 2004; Souktani et al. 2000; Szabo et al. 1998; Wei et al. 2004; Zuo et al. 2009). The inhibitory effect of JAK3 on ClC-2 could, thus, participate in the antiapoptotic effect of the kinase (de Totero et al. 2008; Fainstein et al. 2008; Kim et al. 2010; Nakayama et al. 2009; Uckun et al. 2007).

ClC-2 participates in the regulation of several further functions, such as intracellular chloride concentration and thus cell membrane potential of neurons (Staley et al. 1996), survival of male germ cells and photoreceptors (Bosl et al. 2001), as well as pulmonary chloride and water secretion, which is a prerequisite for fetal lung development (Blaisdell et al. 2000). Whether or not the respective cells do express JAK3 and are thus sensitive to regulation by this kinase remains to be shown.

In conclusion, JAK3 is a powerful kinase inhibiting the cell volume regulatory Cl^- channel ClC-2. In JAK3-expressing cells, the kinase may thus participate in the regulation of cell volume and apoptosis. At least in theory, downregulation of ClC-2 and/or similar Cl^- channels may confer resistance to apoptosis and may thus contribute to the neoplastic effects of the ^{A568V}JAK3 mutation.

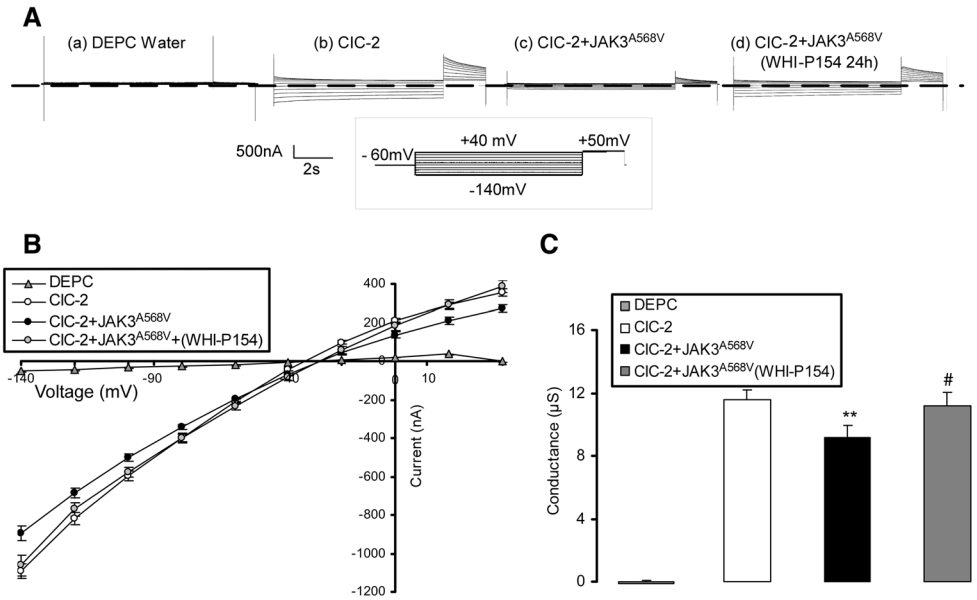

Fig. 3 The effect of ^{A568V}JAK3 is reversed by JAK3 inhibitor WHI-P154 **A**: Representative original tracings showing currents in *Xenopus* oocytes injected with water (a), ClC-2 alone (b), or coexpressing ClC-2 with constitutive active ^{A568V}JAK3 without (c) and with (d) a prior treatment with JAK3 inhibitor WHI-P154 (22 µM) for 24 h **B**: Arithmetic means ± SEM (n = 10–22) of current (I) as a function of the potential difference across the cell membrane (V) in *Xenopus* oocytes injected with water (DEPC water, *gray triangles*), expressing ClC-2 alone (ClC-2, *white circles*) or expressing ClC-2 together with constitutively active ^{A568V}JAK3 and incubated for 24 h in the absence (ClC-2 + ^{A568V}JAK3, *black circles*) or presence of the JAK3 inhibitor WHI-P154 (22 µM) (ClC-2 + ^{A568V}JAK3 + WHI-P154, light gray circles) **C**: Arithmetic means ± SEM (n = 10–22) of the conductance calculated by linear fit of I/V curves shown in B between −140 and −80 mV in *Xenopus* oocytes injected with water (DEPC water, *gray bar*), expressing ClC-2 alone (ClC-2, *white bar*) or expressing ClC-2 together with ^{A568V}JAK3 and incubated for 24 h in the absence (ClC-2 + ^{A568V}JAK3, *black bar*) or presence of WHI-P154 (22 µM, ClC-2 + ^{A568V}JAK3 + WHI-P154, *light gray bar*). ** ($p < 0.01$) indicates statistically significant difference from ClC-2 (i.e., in the absence of JAK3). # ($p < 0.05$) indicates statistically significant difference from the absence of JAK3 inhibitor WHI-P154

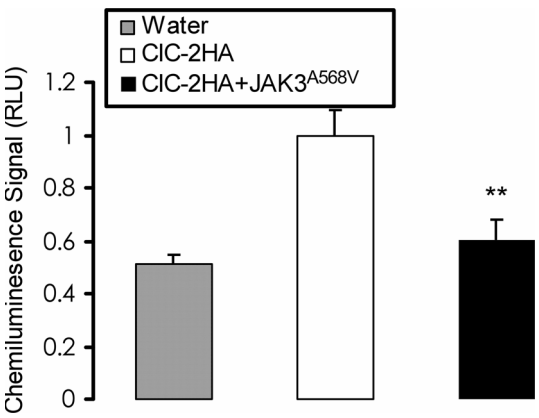

Fig. 4 Effect of ^{A568V}JAK3 on surface ClC-2 protein abundance in ClC-2-HA-expressing *Xenopus* oocytes Arithmetic means ± SEM (n = 82-98) of the chemiluminescence in *Xenopus* oocytes injected with water (DEPC water, *gray bar*), expressing ClC-2-HA alone (ClC-2, *white bar*), or expressing ClC-2-HA together with constitutively active ^{A568V}JAK3 (ClC-2 + ^{A568V}JAK3, *black bar*). ** ($p < 0.001$) indicates statistically significant difference to expression of ClC-2 alone

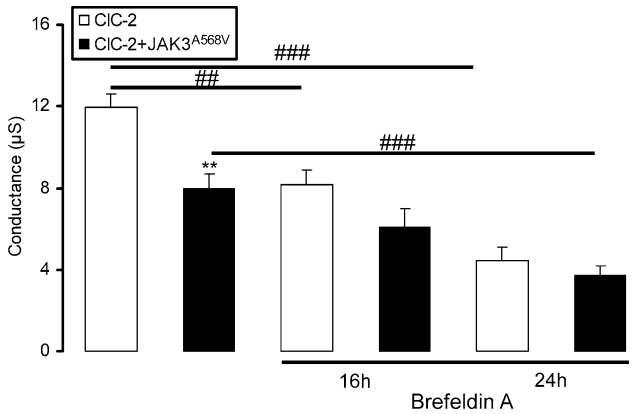

Fig. 5 Effect of brefeldin A on ClC-2 channel activity with or without coexpression of ^{A568V}JAK3. Arithmetic means ± SEM (n = 8–17) of conductance calculated by linear fit of the respective I/V curves between −140 and −80 mV in *Xenopus* oocytes injected with ClC-2 alone (ClC-2, *white bars*) or expressing ClC-2 together with ^{A568V}JAK3 (ClC-2 + ^{A568V}JAK3, *black bars*) prior to (*left bars*) and following incubation with brefeldin A (5 µM) for 16 h (16 h) or 24 h (24 h). ** ($p < 0.01$) indicates statistically significant difference from expression of ClC-2 alone, ### ($p < 0.001$) indicates statistically significant difference from the respective value prior to brefeldin A treatment

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