Original Paper

Regulation of Large Conductance Voltage- and Ca^{2+} -Activated K^+ Channels by the Janus Kinase JAK3

Jamshed Warsi^a Yogesh Singh^a Bernat Elvira^a Zohreh Hosseinzadeh^{a,b}
Florian Lang^a

^aDepartment of Physiology I, University of Tübingen, Tübingen, ^bInstitute for Ophthalmic Research Experimental Retinal Prosthetics, University of Tübingen, Tübingen, Germany

Key Words

Oocytes • Voltage clamp • B lymphocytes • Jak3 knockout mice

Abstract

Background/Aims: Janus kinase 3 (JAK3), a tyrosine kinase contributing to the regulation of cell proliferation and apoptosis of lymphocytes and tumour cells, has been shown to modify the expression and function of several ion channels and transport proteins. Channels involved in the regulation of cell proliferation include the large conductance voltage- and Ca^{2+} -activated K^+ channel BK. The present study explored whether JAK3 modifies BK channel protein abundance and current. **Methods:** cRNA encoding Ca^{2+} -insensitive BK channel ($\text{BK}^{\text{M513I}+\Delta 899-903}$) was injected into *Xenopus* oocytes with or without additional injection of cRNA encoding wild-type JAK3, constitutively active $\text{A}^{568\text{V}}$ JAK3, or inactive $\text{K}^{851\text{A}}$ JAK3. Voltage gated K^+ channel activity was measured utilizing dual electrode voltage clamp. Moreover, BK channel protein abundance was determined utilizing flow cytometry in CD19^+ B lymphocyte cell membranes from mice lacking functional JAK3 (*jak3*^{-/-}) and corresponding wild-type mice (*jak3*^{+/+}). **Results:** BK activity in $\text{BK}^{\text{M513I}+\Delta 899-903}$ expressing oocytes was slightly but significantly decreased by coexpression of wild-type JAK3 and of $\text{A}^{568\text{V}}$ JAK3, but not by coexpression of $\text{K}^{851\text{A}}$ JAK3. The BK channel protein abundance in the cell membrane was significantly higher in *jak3*^{-/-} than in *jak3*^{+/+} B lymphocytes. The decline of conductance in BK and JAK3 coexpressing oocytes following inhibition of channel protein insertion by brefeldin A (5 μM) was similar in oocytes expressing BK with JAK3 and oocytes expressing BK alone, indicating that JAK3 might slow channel protein insertion into rather than accelerating channel protein retrieval from the cell membrane. **Conclusion:** JAK3 is a weak negative regulator of membrane BK protein abundance and activity.

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Introduction

Janus kinase 3 (JAK3) is expressed in several tissues including hematopoietic cells [1-3]. The kinase is involved in the signalling of hematopoietic cell cytokine receptors [4-8]. JAK3 has been shown to stimulate cell proliferation and to inhibit apoptosis of lymphocytes and

Prof. Dr. Florian Lang

Department of Physiology, University of Tübingen,
Gmelinstr. 5, D-72076 Tübingen, (Germany)
Tel. +49 7071/2972194, Fax +49 7071/295618, E-Mail florian.lang@uni-tuebingen.de

tumour cells [9-13]. The gain of function mutation ^{A572V}JAK3 is found in acute megakaryoblastic leukemia cells [14, 15]. Kinase activity of JAK3 is disrupted by replacement of the ATP coordinating lysine in the catalytic subunit with alanine thus yielding the inactive ^{K855A}JAK3 [16].

Ion channels implicated in the regulation of cell proliferation include the large conductance Ca²⁺-activated K⁺ channels (maxi K⁺ channel or BK channels) [17-29]. The present study thus explored, whether JAK3 modifies BK channel activity. To this end, the Ca²⁺-insensitive BK channel (BK^{M513I+Δ899-903}) was expressed in *Xenopus* oocytes without or with additional expression of wild type JAK3, constitutively active ^{A568V}JAK3, or inactive ^{K851A}JAK3. In those oocytes the voltage gated K⁺ current was determined utilizing dual electrode voltage clamp. The *Xenopus* oocytes allow strong expression and functional analysis of human channels with large signal to noise ratio. In order to test the significance of the observations in *Xenopus* oocytes for the channel regulation in mammalian cells, BK protein abundance was quantified in B lymphocytes isolated from gene targeted mice lacking functional JAK3 (*jak3*^{-/-}) and from corresponding wild-type mice (*jak3*).

Materials and Methods

Ethical Statement

All experiments conform to the 'European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes' (Council of Europe No 123, Strasbourg 1985) and were conducted according to the German law for the welfare of animals. All procedures were reviewed and approved by the respective government authority of the state Baden-Württemberg (Regierungspräsidium) prior to the start of the study.

Constructs

Constructs encoding mouse Ca²⁺-insensitive BK channel (BK^{M513I+Δ899-903}) [29, 30] (kindly provided by J Lingle), and/or mouse wild-type JAK3, inactive ^{K851A}JAK3 mutant and gain of function ^{A568V}JAK3 mutant [31], were used for generation of the respective cRNA as described previously [32-34].

Voltage clamp in *Xenopus* oocytes

Xenopus oocytes were prepared as previously described [35]. 20 ng cRNA encoding BK and 10 ng of cRNA encoding wild-type JAK3, constitutively active ^{A568V}JAK3 or inactive ^{K851A}JAK3 kinase were injected on the same day after preparation of the oocytes [36-38]. The oocytes were maintained at 17°C in ND96, a solution containing (in mM): 88.5 NaCl, 2 KCl, 1 MgCl₂, 1.8 CaCl₂, 5 HEPES, 5 Sodium pyruvate (C₃H₃NaO₃). Gentamycin (100 mg/l), Tetracycline (50 mg/l), Ciprofloxacin (1.6 mg/l), and Theophylline (90 mg/l) were added and pH adjusted to 7.4 [39-41]. Where indicated, brefeldin A (5 μM) was added to the respective solutions. The voltage clamp experiments were performed at room temperature 3 days after the first injection. BK channel currents were elicited every 1 s with 1 s pulses from -150 to +190 mV in 2 s increments of 20 mV steps from a holding potential of -60 mV. The data were filtered at 2 kHz and recorded with a Digidata A/D-D/A converter (1322A Axon Instruments) and Clampex 9.2 software for data acquisition and analysis (Axon Instruments) [42-44]. The control superfusate (ND96) contained (in mM): 93.5 NaCl, 2 KCl, 1.8 CaCl₂, 1 MgCl₂, 2.5 NaOH and 5 HEPES (pH 7.4). The flow rate of the superfusion was approx. 20 ml/min, and a complete exchange of the bath solution was reached within about 10 s [45-47].

Mice

CD19⁺ B cells were used for the BK channel protein expression experiments from 8-12 weeks old female gene-targeted mice lacking functional JAK3 (*jak3*^{-/-}) and in age- and sex-matched wild type mice (*jak3*^{+/+}) (Charles River, Sulzfeld, Germany) [48]. The mice were obtained from the Jackson laboratory (Bar Harbor, ME, USA) and had free access to water and control food (SSniff, Soest, Germany).

Flow cytometry of BK channel surface protein abundance in lymphocytes

To stain the CD19⁺ B cells, spleen and lymph nodes were collected from the mice and macerated using syringe plunger. Cell suspension was centrifuged at 600 x g at 4°C for 5 minutes and cells pellet was

treated with RBC lysis buffer for 1 minute and then washed for three times with 10% RPMI1640 media. After washing, 1×10^6 cells were stained with 0.5 μg antibodies per sample [original concentrations; 0.2 $\mu\text{g}/\mu\text{l}$ anti-CD4-APC (eBioscience, Germany), anti-CD19-PE (eBioscience, Germany) and BK rabbit anti-mouse antibodies (alomone labs, Israel)] in 50 μl 1 x DPBS (Sigma, Germany) for 30 minutes in dark and washed the cells. After washing the cells 0.2 μl Goat anti-Rabbit IgG-FITC (eBioscience Germany) in 50 μl of 1 x DPBS was added and incubated for another 30 minutes in the dark. Finally, the cells were washed twice with 1 x DPBS and added 200 μl of DPBS. All the washing steps were performed at 600 x g for 5 minutes and at room temperature. Cells were immediately acquired using BD FACSCalibre™ (BD Bioscience, Heidelberg, Germany) flow cytometry and data were analysed by Flowjo (Treestar, USA).

Statistical analysis

Data are provided as means $\pm$ SEM, n represents the number of oocytes or of cell preparations investigated. As different batches of oocytes may yield different results, comparisons were always made within a given oocyte batch. All voltage clamp experiments were repeated with at least 3 batches of oocytes; in all repetitions qualitatively similar data were obtained. Data were tested for significance using ANOVA (Tukey test or Kruskal-Wallis test) or t-test, as appropriate. Results with $p < 0.05$ were considered statistically significant.

Results

The present study addressed a putative influence of Janus activated kinase JAK3 on large conductance voltage- and Ca^{2+} -activated K^+ channel BK. In a first series of experiments cRNA encoding Ca^{2+} -insensitive BK channel ($\text{BK}^{\text{M513I}+\Delta 899-903}$) was injected into *Xenopus* oocytes with or without additional injection of cRNA encoding JAK3. The voltage gated K^+ current was determined by dual electrode voltage clamp experiments. As shown in Fig. 1, voltage gated current was negligible in water injected oocytes indicating that oocytes did not express

Fig. 1. Co-expression of wild-type JAK3 decreased the K^+ current in BK expressing *Xenopus* oocytes. (A) Representative original tracings showing currents in *Xenopus* oocytes injected with water (a), expressing BK alone (b) or expressing BK with additional co-expression of wild-type JAK3 (c). The voltage protocol is shown (not to scale). Currents were activated by depolarization from -150 to +190 mV from a holding potential of -60 mV. (B) Arithmetic means $\pm$ SEM (n = 44-57) of the current (I) as a function of the potential difference across the cell membrane (V) in *Xenopus* oocytes injected with water (white circles) or expressing BK without (white squares) or with (white triangles) additional co-expression of wild-type JAK3. C: Arithmetic means $\pm$ SEM (n = 44-57) of the conductance calculated by linear fit of I/V-curves shown in B between 130 mV and 190 mV in *Xenopus* oocytes injected with water (striped bar), or expressing BK without (white bar) or with (black bar) additional co-expression of wild-type JAK3. * ($p < 0.05$) indicates statistically significant difference from oocytes expressing BK alone.

Fig. 2. The effect of wild-type JAK3 on BK was mimicked by constitutively active A^{568V} JAK3 but not by inactive K^{851A} JAK3. (A) Representative original tracings showing currents in *Xenopus* oocytes injected with water (a) expressing BK alone (b), expressing BK together with constitutively active A^{568V} JAK3 (c), or expressing BK with inactive K^{851A} JAK3 (d). (B) Arithmetic means $\pm$ SEM ($n = 15$ –32) of the current (I) as a function of the potential difference across the cell membrane (V) in *Xenopus laevis* oocytes injected with water (white circles), expressing BK alone (white squares) or expressing BK together with constitutively active A^{568V} JAK3 (white triangles) or inactive K^{851A} JAK3 (white diamonds). (C) Arithmetic means $\pm$ SEM ($n = 15$ –32) of the conductance calculated by linear fit of I/V -curves shown in B between 130 mV and 190 mV in *Xenopus* oocytes injected with water (striped bar), expressing BK alone (white bar), expressing BK with constitutively active A^{568V} JAK3 (black bar), or expressing BK with inactive K^{851A} JAK3 (grey bar). ** ($p < 0.01$) indicates statistically significant difference from oocytes expressing BK alone. # ($p < 0.05$) indicates statistically significant difference from oocytes expressing BK and A^{568V} JAK3.

endogenous voltage gated K^+ channels. In contrast, large voltage gated K^+ currents were observed in oocytes injected with cRNA encoding $BK^{M513I+\Delta 899-903}$. The additional injection of cRNA encoding wild-type JAK3 was followed by a slight but significant decrease of the voltage gated current.

As illustrated in Fig. 2, the effect of wild-type JAK3 was mimicked by the constitutively active A^{568V} JAK3. In $BK^{M513I+\Delta 899-903}$ expressing oocytes, the additional injection of cRNA encoding A^{568V} JAK3 was followed by a slight but significant decrease of the voltage gated current. In contrast, coexpression of the inactive JAK3 mutant K^{851A} JAK3 did not significantly modify voltage gated current in $BK^{M513I+\Delta 899-903}$ expressing oocytes.

An additional series of experiments addressed whether JAK3 impacts on BK protein abundance in mammalian hematopoietic cells. To this end, BK channel protein abundance was determined utilizing flow cytometry in the cell membrane of B lymphocytes from mice lacking functional JAK3 ($jak3^{-/-}$) and from corresponding wild-type mice ($jak3^{+/+}$). As illustrated in Fig. 3, the BK channel protein abundance was significantly higher in B lymphocytes from $jak3^{-/-}$ mice than in B lymphocytes isolated from $jak3^{+/+}$ mice.

JAK3 could decrease BK 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, BK and JAK3 expressing *Xenopus* oocytes were treated with 5 μ M brefeldin A, a substance disrupting insertion of new channel protein into the cell

Fig. 3. BK channel protein abundance was enhanced in the cell membrane of B lymphocytes isolated from JAK3 deficient mice. (A, B): Original dot blots of CD19 and CD4 abundance in the cell membrane of lymphocytes isolated from *jak3^{+/+}* (A) and *jak3^{-/-}* (B) mice. (C, D): Original dot blots of CD19 and BK channel protein abundance in the cell membrane of lymphocytes from *jak3^{+/+}* (C) and *jak3^{-/-}* (D) mice. E: Arithmetic means $\pm$ SEM (n = 3) of BK channel protein abundance in the plasma membrane of B lymphocytes from *jak3^{+/+}* (white bar) and *jak3^{-/-}* (black bar) mice. ** (p<0.01) indicates statistically significant difference from *jak3^{+/+}* lymphocytes.

Fig. 4. Effect of brefeldin A on BK channel activity with or without co-expression of JAK3. Arithmetic means $\pm$ SEM (n = 16-30) of conductance calculated by linear fit of respective I/V-curves between 20 mV and 50 mV in *Xenopus* oocytes injected with BK alone (BK, white bars) or expressing BK together with JAK3 (black bars) prior to (left bars, 0h) and following incubation with brefeldin A (5 μ M) for 16 h (16 h) or 24 h (24 h). * (p<0.05) indicates statistically significant difference from expression of BK alone, ## and ### (p<0.01, p<0.001) indicates statistically significant difference from respective value prior to brefeldin A treatment.

membrane. As illustrated in Fig. 4, the decline of conductance in the presence of brefeldin A was similar in oocytes expressing BK together with JAK3 and oocytes expressing BK alone.

Discussion

The present study identifies a novel effect of Janus activated kinase JAK3, i.e. the slight but statistically significant down-regulation of the large conductance voltage- and Ca^{2+} -

activated K⁺ channel BK. Coexpression of wild type JAK3 or of the gain of function mutant ^{A568V}JAK3 decreased the voltage gated current in *Xenopus* oocytes expressing Ca²⁺-insensitive BK channel (BK^{M513I+Δ899-903}). Moreover, BK channel protein abundance was significantly higher in B lymphocytes from *jak3*^{-/-} mice than in B lymphocytes from *jak3*^{+/+} mice. Thus, JAK3 sensitive regulation of BK in oocytes presumably reflects a similar regulation of BK channels in mammalian cells. Apparently, JAK3 is at least in part effective by decreasing the channel protein abundance in the cell membrane.

It must be kept in mind, though, that the effect of JAK3 on BK surface expression and activity does not necessarily reflect a direct phosphorylation of the BK channel protein. Instead, JAK3 may modify BK channel expression and activity indirectly. For instance, JAK3 may phosphorylate regulators of the channel protein thus indirectly modifying its regulation. Moreover, JAK3 is a powerful inhibitor of the Na⁺/K⁺ ATPase activity [49]. JAK3 expression is up-regulated [50] and activated [51] upon hypoxia and JAK3 is activated by energy depletion [52]. The isoform JAK2 has similarly been shown to be a powerful negative regulator of Na⁺/K⁺ ATPase [53]. Inhibition of Na⁺/K⁺ ATPase activity is in turn known to down-regulate K⁺ channels [54-56].

Moreover, the difference between B lymphocytes from *jak3*^{-/-} mice and *jak3*^{+/+} mice may be affected by an influence of JAK3 deficiency on the abundance of inflammatory cells and the release of inflammatory mediators modifying BK channel activity. Along those lines enhanced serum calcitriol and FGF23 levels have been observed in *jak3*^{-/-} mice [57]. Moreover, JAK3-deficient mice are volume depleted [48].

To which extent the slight effect of JAK3 on BK channel protein abundance and BK channel activity modifies BK channel sensitive cellular functions, remains to be determined. In theory, inhibition of K⁺ channels could counteract cell shrinkage and apoptosis by counteracting cellular loss of K⁺ ions [58]. However, activation rather than inhibition of K⁺ channels fosters cell proliferation [59-61]. Stimulation of large conductance Ca²⁺-activated K⁺ channels (maxi K⁺ channel or BK channels) has thus been observed in proliferating cells [17-29]. The stimulating effect of JAK3 on cell proliferation [9-13] is thus hardly supported by inhibition of BK channels. Clearly, additional experimental effort is needed to clarify the functional significance of JAK3 sensitive BK channel abundance and activity.

In conclusion, wild-type JAK3 and constitutively active ^{A568V}JAK3 slightly but significantly down-regulate the large conductance voltage- and Ca²⁺-activated K⁺ channel BK.

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Disclosure Statement

The authors of this manuscript state that they do not have any conflict of interests and nothing to disclose.

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