J Membrane Biol (2014) 247:331–338
DOI 10.1007/s00232-014-9636-1

Down-Regulation of the Epithelial Na^+ Channel ENaC by Janus kinase 2

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Received: 14 November 2013 / Accepted: 29 January 2014 / Published online: 23 February 2014

Abstract Janus kinase-2 (JAK2), a signaling molecule mediating effects of various hormones including leptin and growth hormone, has previously been shown to modify the activity of several channels and carriers. Leptin is known to inhibit and growth hormone to stimulate epithelial Na^+ transport, effects at least partially involving regulation of the epithelial Na^+ channel ENaC. However, no published evidence is available regarding an influence of JAK2 on the activity of the epithelial Na^+ channel ENaC. In order to test whether JAK2 participates in the regulation of ENaC, cRNA encoding ENaC was injected into *Xenopus* oocytes with or without additional injection of cRNA encoding wild type JAK2, gain-of-function ^{V617F}JAK2 or inactive ^{K882E}JAK2. Moreover, ENaC was expressed with or without the ENaC regulating ubiquitin ligase Nedd4-2 with or without JAK2, ^{V617F}JAK2 or ^{K882E}JAK2. ENaC was determined from amiloride (50 μM)-sensitive current (I_{amil}) in dual electrode voltage clamp. Moreover, I_{amil} was determined in colonic tissue utilizing Ussing chambers. As a result, the I_{amil} in ENaC-expressing oocytes was significantly decreased following coexpression of JAK2 or ^{V617F}JAK2, but not by coexpression of ^{K882E}JAK2. Coexpression of JAK2 and Nedd4-2 decreased I_{amil} in ENaC-expressing oocytes to a larger extent than coexpression of Nedd4-2 alone. Exposure of ENaC- and JAK2-expressing oocytes to JAK2 inhibitor AG490 (40 μM) significantly increased I_{amil}. In colonic epithelium, I_{amil} was significantly enhanced by AG490 pretreatment (40 μM, 1 h). In conclusion, JAK2 is a powerful inhibitor of ENaC.

Keywords JAK2 · Amiloride · JAK2 inhibitor AG490 · Nedd4-2

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Introduction

The Janus-activated kinases (JAK) are a family of non-receptor tyrosine kinases containing two kinase homology domains (Yeh and Pellegrini 1999). They are activated by a variety of cytokines and play decisive roles in development, differentiation, and host defense (Yeh and Pellegrini 1999).

JAK2 mediates the cellular effects of several hormones and cytokines (Lopez et al. 2010; Noon-Song et al. 2011; Spivak 2010), such as leptin (Morris and Rui 2009; Schmid et al. 2012), growth hormone (Brooks and Waters 2010; Xia et al. 2002; Yang et al. 2010), erythropoietin (Spivak 2010), thrombopoietin (Spivak 2010), and granulocyte colony-stimulating factor (Spivak 2010). JAK2 is in addition activated by oxidative stress & ischemia (Kurdi and Booz 2009), energy depletion (Bhavsar et al. 2013), and hypertonicity (Garnovskaya et al. 2003; Gatsios et al. 1998). JAK2 participates in the signaling of cell proliferation and the gain-of-function mutation ^{V617F}JAK2 leads to the development of myeloproliferative malignancy (Shen et al. 2010; Venkitachalam et al. 2012; Yao et al. 2010; Mahfouz et al. 2011). Accordingly, JAK2 inhibitors are considered for the treatment of myeloproliferative disorders (Baskin et al. 2010; Ho et al. 2010; Oh and Gotlib 2010; Pardanani et al. 2011; Santos and Verstovsek 2011; Tefferi 2010).

JAK2 has further been shown to participate in the regulation of carriers and ion channels. Channels reported to be JAK2 sensitive include Cl^- channels (Selvaraj et al. 2000; Hosseinzadeh et al. 2012a) K^+ channels (Deachapunya et al. 2012; Hosseinzadeh et al. 2013), Ca^{2+} channels (Dorkkam et al. 2013) and TRP channels (Qiu et al. 2010). Carriers shown to be regulated by JAK2 include facilitative glucose transporters (Gong et al. 1998; Yokota et al. 1998), Na^+ coupled glucose transport (Hosseinzadeh et al. 2011a), Na^+ coupled neutral amino acid transporter B(0)AT (SLC6A19) (Bhavsar et al. 2011), Na^+ coupled glutamate transport (Hosseinzadeh et al. 2011b), Na^+ and Cl^- coupled transport of betaine and GABA (Hosseinzadeh et al. 2012b), Na^+/H^+ exchanger (Coaxum et al. 2009) as well as Na^+, K^+, $2Cl^-$ cotransport (Selvaraj et al. 2000). Thus, JAK2 is a powerful regulator of transport processes. Specifically, JAK2 has been shown to modify transport in colonic epithelia (Deachapunya et al. 2012).

The present study thus explored whether JAK2 modifies the activity of the epithelial Na^+ channel ENaC, which accomplishes apical Na^+ entry in several epithelia including colonic epithelium (Kunzelmann and Mall 2002; Rossier et al. 2002) and may participate in regulatory cell volume increase following cell shrinkage (Bohmer et al. 2000; Ross et al. 2007). To this end, cRNA encoding ENaC was injected into *Xenopus* oocytes with or without cRNA encoding wild type JAK2, gain-of-function ^{V617F}JAK2 or inactive ^{K882E}JAK2. ENaC-mediated current was estimated from amiloride-sensitive outward current utilizing dual electrode voltage clamp. In addition, amiloride-sensitive current across colonic epithelia without or with prior treatment with JAK2 inhibitor AG490 was determined using Ussing chamber.

Materials and Methods

All animal experiments were conducted according to the German law for the welfare of animals and were reviewed, and approved by the respective government authority of the state Baden-Württemberg (Regierungspräsidium) prior to the start of the study. Experiments were performed in tissues from C57BL/6 female wild type mice (age 6 months). Mice had free access to control diet (Walkermühle, Hechingen, Germany) and tap drinking water ad libitum. To obtain tissue, the animals were killed by cervical dislocation.

Constructs

For generating cRNA (Henrion et al. 2012), constructs encoding mouse ENaC (Friedrich et al. 2003), wild type JAK2, inactive ^{K882E}JAK2, and gain-of-function ^{V617F}JAK2 (Hosseinzadeh et al. 2011a, b) were used as described previously (Almilaji et al. 2013a; Pakladok et al. 2013).

Voltage Clamp in *Xenopus* Oocytes

Xenopus oocytes were prepared as previously described (Alesutan et al. 2012; Henrion et al. 2012). When not indicated otherwise, 1 ng/subunit cRNA encoding wild type α, β, γENaC were injected on the first day, and 10 ng of cRNA encoding wild type JAK2, ^{V617F}JAK2, ^{K882E}JAK2 or Nedd4-2 were injected on the same or second day after preparation of the oocytes (Hosseinzadeh et al. 2012a; Pathare et al. 2012). The oocytes were maintained at 17 °C in ND96 solution containing (in mM): 96 NaCl, 2 KCl, 1 $MgC1_2$, 1.8 $CaC1_2$, 5 HEPES/NaOH, pH 7.4, supplemented with tetracycline (50 mg/l), ciprofloxacin (1.6 mg/l), refobacin (100 mg/l), theophiline (90 mg/l), and 2.5 mM sodium pyruvate. Where indicated, the JAK2 inhibitor AG490 (40 μM) was added to the respective solutions. All experiments were performed at room temperature 3 days after injection. In two-electrode voltage-clamp experiments amiloride (50 μM)-sensitive currents were determined at a holding potential of -80 mV. The data were filtered at 1 kHz and recorded with a Digidata A/D–D/A converter, and Clampex 9.2 software for data acquisition and analysis (Axon Instruments) (Almilaji et al. 2013b). The data were analyzed using Clampfit 9.2 (Axon Instruments) software (Hosseinzadeh et al. 2012a; Pathare et al. 2012).

Ussing Chamber Experiments

ENaC activity was estimated from the amiloride-sensitive potential difference and current across the colonic epithelium. After removing the outer serosal and the muscular layer of late distal colon under a microscope, tissues were mounted onto a custom-made mini-Ussing chamber with an opening diameter of 0.99 mm, and an opening area of 0.00769 cm^2. Transepithelial potential difference (V_{te}) was determined continuously, and transepithelial resistance (R_{te}) estimated from the voltage deflections (ΔV_{te}) elicited by imposing rectangular test currents of 1 μA and 1.2 s duration at a rate of 8/min. R_{te} may be an underestimate of the real transepithelial resistance (Rexhepaj et al. 2006). The calculation of short circuit currents from $\Delta V_{te,\ amiloride}/R_{te}$ may be biased accordingly and yield only an apparent current (I_{amil}). Nevertheless, the same chamber has been used prior to and following the JAK2 inhibitor, and the experiments still allow uncovering alterations of ENaC activity (Rexhepaj et al. 2006). The serosal and luminal perfusate contained (in mM): 145 NaCl, 1 $MgCl_2$, 2.6 Ca-gluconate, 0.4 KH_2PO_4, 1.6 K_2HPO_4, and 5 glucose. To assess ENaC-mediated transport, 50 μM amiloride (Sigma, Taufkirchen; in DMSO) was added to the luminal perfusate.

Statistical Analysis

Data are provided as mean ± SEM, n represents the number of oocytes or epithelial tissues investigated. All voltage clamp 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 unpaired *t* test, as appropriate. Results with $p < 0.05$ were considered statistically significant.

Results

The present study explored whether JAK2 participates in the regulation of the epithelial Na^+ channel ENaC. To this end, cRNA encoding ENaC was injected into *Xenopus* oocytes with or without additional injection of cRNA encoding JAK2. In oocytes expressing ENaC, but not in oocytes injected with water, amiloride (50 μM)-sensitive current was recorded, which was significantly decreased by the additional injection of cRNA encoding wild type JAK2 (Fig. 1a, b).

The effect of wild type JAK2 on amiloride-sensitive current was mimicked by the gain-of-function mutant ^{V617F}JAK2, but not by the inactive mutant ^{K882E}JAK2 (Fig. 2). Accordingly, the amiloride-sensitive current was significantly lower in *Xenopus* oocytes expressing ENaC together with ^{V617F}JAK2 than in *Xenopus* oocytes expressing ENaC alone. In contrast, the amiloride-sensitive current in *Xenopus* oocytes expressing ENaC together with ^{K882E}JAK2 was not significantly different from the amiloride-sensitive current in *Xenopus* oocytes expressing ENaC alone (Fig. 2a, b).

A powerful regulator of ENaC is the ubiquitin ligase Nedd4-2. Thus, experiments were performed in ENaC-expressing oocytes without or with coexpression of Nedd4-2 and/or JAK2, ^{V617F}JAK2 or ^{K882E}JAK2. As a result, the decline of amiloride-sensitive current in ENaC-expressing Xenopus oocytes was significantly larger following coexpression of ^{V617F}JAK2 than following coexpression of Nedd4-2 alone or together with ^{K882E}JAK2. Moreover, the amiloride-sensitive current in ENaC and Nedd4-2 expressing oocytes was significantly decreased by the additional coexpression of JAK2 or ^{V617F}JAK2 (Fig. 3a, b).

A further series of experiments explored whether the amiloride-sensitive current in ENaC- and JAK2-expressing *Xenopus* oocytes is sensitive to the pharmacological JAK2 inhibitor AG490. To this end the current in ENaC- and JAK2-expressing *Xenopus* oocytes was determined without and with prior treatment of the oocytes with AG490 (40 μM). As illustrated in Fig. 4, exposure to the inhibitor was followed by an increase of the amiloride-sensitive current, an effect reaching statistical significance within 24 h of preincubation with AG490.

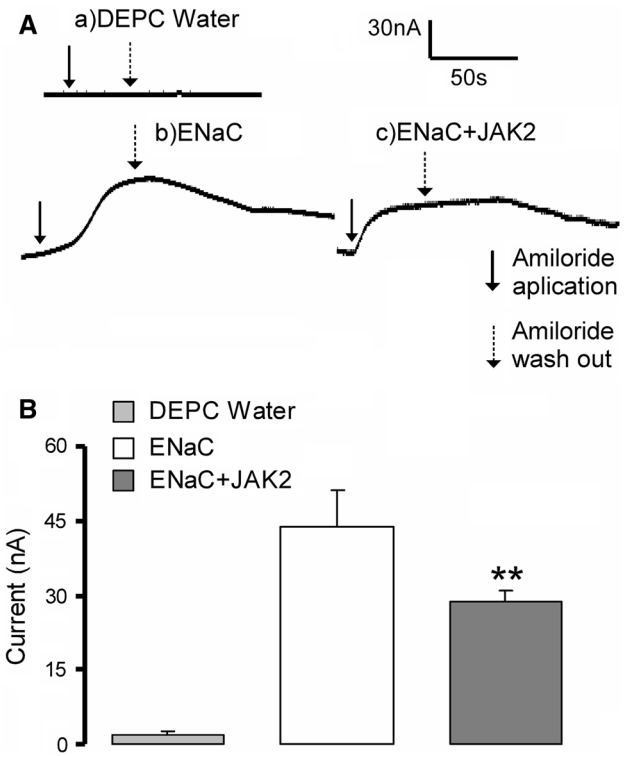

Fig. 1 Effect of wild type JAK2 coexpression on amiloride-sensitive current in ENaC-expressing *Xenopus* oocytes. **a** Original tracings of the amiloride (50 μM)-sensitive current measured at a holding potential of −80 mV in *Xenopus* oocytes injected with water (*a* DEPC water), expressing ENaC alone (*b* ENaC) or expressing ENaC with additional coexpression of wild type JAK2 (*c* ENaC+Jak2). **b** Arithmetic mean values ± SEM ($n = 7$) of the amiloride (50 μM)-sensitive current at −80 mV in *Xenopus* oocytes injected with water (*light gray* DEPC water), expressing ENaC alone (*white bar* ENaC) or expressing ENaC with additional coexpression of wild type JAK2 (*dark gray bar* ENaC+JAK2). ** $p < 0.01$ indicates statistically significant difference from the values obtained in oocytes expressing ENaC alone

In order to test, whether JAK2 participates in the regulation of ENaC in colonic epithelium, amiloride-sensitive current across the murine colonic tissue was evaluated in Ussing chamber experiments without or with pretreatment with the JAK2 inhibitor AG490 (40 μM, 1 h). As illustrated in Fig. 5a, b, the amiloride-sensitive current was significantly increased by the pretreatment of the colonic tissue with AG490 (40 μM, 1 h).

Discussion

The present study discloses a completely novel regulator of the epithelial Na^+ channel ENaC. Coexpression of the JAK2 significantly decreased the amiloride-sensitive current in both, ENaC-expressing *Xenopus* oocytes and

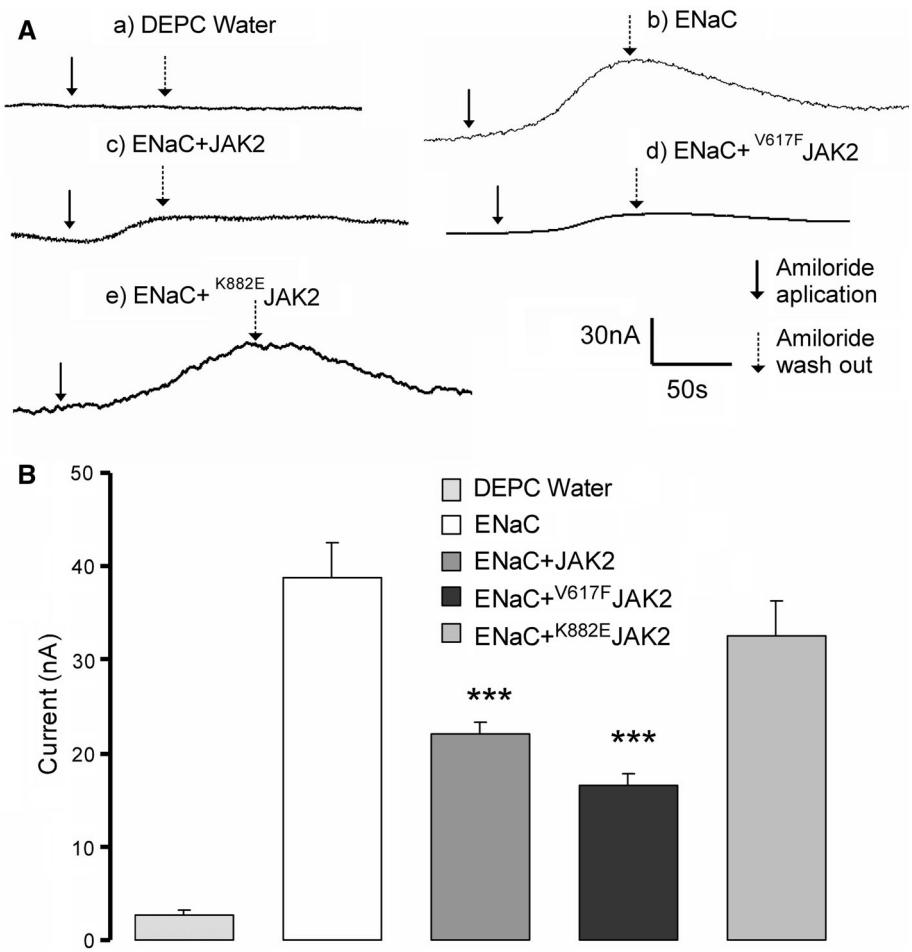

Fig. 2 Effect of gain-of-function mutant ^{V617F}JAK2 and of inactive ^{K882E}JAK2 on amiloride-sensitive current in ENaC-expressing *Xenopus* oocytes. **a** Original tracings of the amiloride (50 μM)-sensitive current at −80 mV holding potential in *Xenopus* oocytes injected with water (*a* DEPC water), expressing ENaC either alone (*b* ENaC) or with additional coexpression of wild type JAK2 (*c* ENaC+JAK2), active ^{V617F}JAK2 (*d* ENaC+^{V617F}JAK2) or inactive ^{K882E}JAK2 (*e* ENaC+^{K882E}JAK2). **b** Arithmetic mean values ± SEM (n = 15–20) of the amiloride (50 μM)-sensitive current at −80 mV holding potential in *Xenopus* oocytes injected with water (*light gray* DEPC water), expressing ENaC alone (*white bar* ENaC), expressing ENaC with additional coexpression of wild type JAK2 (*dark gray bar* ENaC+JAK2), active ^{V617F}JAK2 (*black bar* ENaC+^{V617F}JAK2), or expressing ENaC with additional coexpression of kinase-dead mutant ^{K882E}JAK2 (*light gray bar* ENaC+^{K882E}JAK2). *** $p < 0.001$ indicate statistically significant difference from the values obtained in oocytes expressing ENaC alone

colonic epithelium of the mouse. In *Xenopus* oocytes, the effect of wild type JAK2 was mimicked by the gain-of-function mutant ^{V617F}JAK2, but not by the inactive ^{K882E}JAK2. The effect of ^{V617F}JAK2 on the amiloride-sensitive current in ENaC-expressing Xenopus oocytes was reversed by the JAK2 inhibitor AG490. Accordingly, AG490 significantly enhanced the amiloride-sensitive current in colonic epithelium. The effect of the JAK2 inhibitor AG490 was slow, and JAK2 may modify channel activity indirectly.

The decline of the amiloride-sensitive current in ENaC-expressing oocytes was stronger following coexpression of JAK2 or ^{V617F}JAK2 than following coexpression of the ubiquitin ligase Nedd4-2, a well-known powerful regulator of ENaC (Snyder 2009; Kamynina and Staub 2002). Thus, JAK2 is an even more powerful regulator of ENaC. The additional coexpression of JAK2 or ^{V617F}JAK2 in ENaC and Nedd4-2 expressing oocytes was followed by a further decrease of amiloride-sensitive currents. JAK2 may thus be effective by mechanisms other than or in addition to stimulation of Nedd4-2. It must be kept in mind, though, that at least in theory, the activity of coexpressed Nedd4-2 may be modulated by JAK2.

The JAK2 sensitivity of ENaC could contribute to the well-described natriuretic effects of leptin (Villarreal et al. 1998, 2006), a hormone known to stimulate JAK2 (Morris and Rui 2009; Schmid et al. 2012). Leptin stimulates the generation of nitric oxide (Beltowski et al. 2010), which

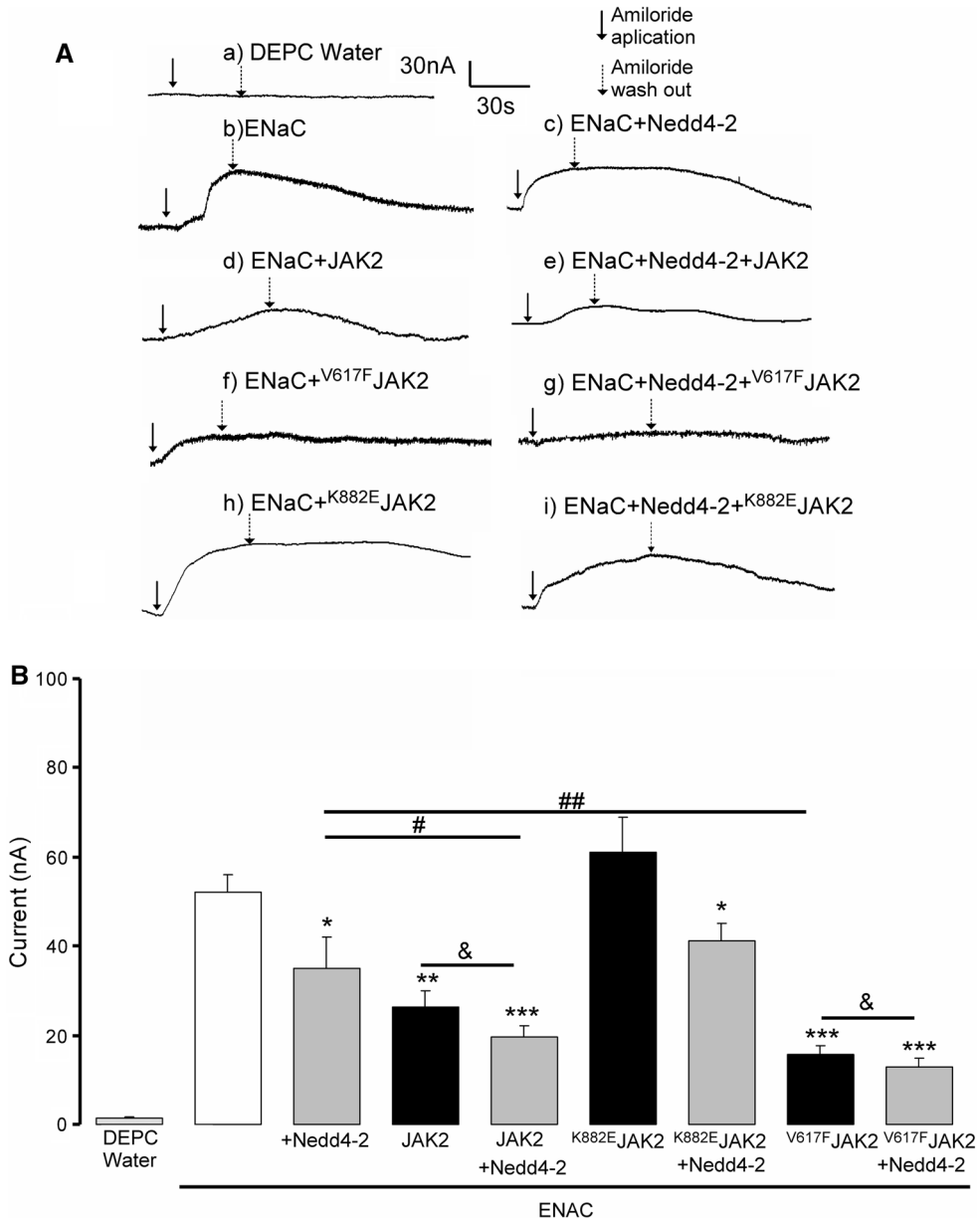

Fig. 3 Effect of JAK2 and of ^{V617F}JAK2 in the presence and absence of Nedd4-2 on amiloride-sensitive current in ENaC-expressing *Xenopus* oocytes. **a** Original tracings of the amiloride (50 μM)-sensitive current at −80 mV holding potential in *Xenopus* oocytes injected with water (*a* DEPC water), expressing ENaC either alone (*b* ENaC) or with additional coexpression of Nedd4-2 (*c* ENaC+Nedd4-2), JAK2 (*d* ENaC+JAK2), both JAK2 and Nedd4-2 (*e* ENaC+Nedd4-2+JAK2), ^{V617F}JAK2 (*f* ENaC+^{V617FE}JAK2), both Nedd4-2 and ^{V617F}JAK2 (*g* ENaC+Nedd4-2+^{V617F}JAK2), ^{K882E}JAK2 (*h* ENaC+^{K882E}JAK2) or both ^{K882E}JAK2 and Nedd4-2 (*i* ENaC+Nedd42+^{K882E}JAK2). **b** Arithmetic mean values ± SEM (n = 15–25) of the amiloride (50 μM)-sensitive current at −80 mV holding potential in *Xenopus* oocytes injected with water (*light gray* DEPC water), expressing ENaC alone (*white bar* ENaC), expressing ENaC with additional coexpression of Nedd4-2 (*gray bar*), or of JAK2, ^{V617F}JAK2 or ^{K882E}JAK2 either alone (*black bars*) or together with Nedd4-2 (*gray bars*). *, **, *** $p < 0.05$, $p < 0.01$, $p < 0.001$ indicate statistically significant difference from the value obtained in oocytes expressing ENaC alone, $^{\#,\#\#}$ $p < 0.05$, $p < 0.01$ indicate statistically significant difference from the respective value with Nedd4-2 expression alone, & $p < 0.05$ indicate statistically significant difference from the value expressing JAK2 or ^{V617F}JAK2 alone

has similarly been shown to downregulate ENaC (Helms et al. 2005). At least in some cells, nitric oxide formation depends on JAK2 (Han et al. 2006). Notably, the natriuretic effect of leptin was abolished by pharmacological inhibitors of phosphoinositide 3-kinase (PI3K) (Beltowski et al. 2010), a surprising finding in view of the PI3K-dependent activation of the serum & glucocorticoid inducible kinase SGK1, a powerful stimulator of ENaC and

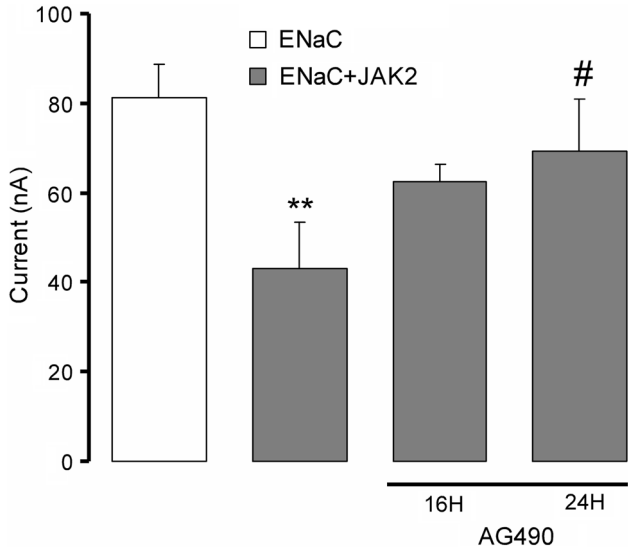

Fig. 4 The JAK2 inhibitor AG490 increased the current in ENaC- and JAK2-expressing *Xenopus* oocytes. Arithmetic mean values ± SEM ($n = 12$–15) of the amiloride (50 μM)-sensitive current at −80 mV holding potential in *Xenopus* oocytes expressing ENaC alone (*white bar* ENaC) or expressing ENaC and active JAK2 (ENaC+JAK2) without or with (*light gray bars*) 16–24 h pretreatment with JAK2 inhibitor AG490 (40 μM). ** $p < 0.01$ indicates statistically significant difference from the values obtained in oocytes expressing ENaC, and $^{\#}$ $p < 0.05$ indicates statistically significant difference from the absence of JAK2 inhibitor AG490 (40 μM) in oocytes expressing ENaC+JAK2

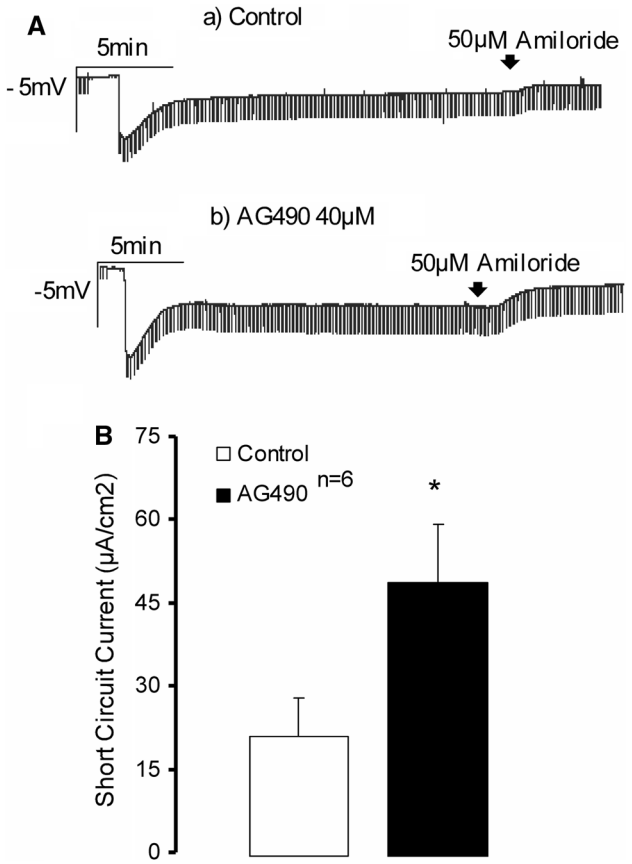

Fig. 5 Effect JAK2 inhibitor AG490 on amiloride-sensitive current in colonic epithelium. **a** Original tracings from typical experiments illustrating the effect of amiloride (50 μM) on the transepithelial potential difference of colonic segments without (*a*) or with (*b*) pretreatment with JAK2 inhibitor AG490 (40 μM). **b** Arithmetic mean values ± SEM ($n = 6$) of amiloride (50 μM)-induced current across colonic epithelium in the absence (*white bar* control) or presence (*black bar*) of JAK2 inhibitor AG490. * indicates statistically significant ($p < 0.05$) difference from the absence of inhibitor

mediator of insulin induced renal salt retention and hypertension (Lang et al. 2006). PI3K is required for the JAK2-dependent membrane binding of the multisite docking protein Gab1 (Wolf et al. 2013), an observation pointing to interaction of PI3K signaling and JAK2 signaling. Leptin resistance of natriuresis may contribute to hypertension in obesity (Beltowski et al. 2010; Coatmellec-Taglioni and Ribiere 2003). It would thus be of interest to learn, whether leptin resistance involves decreased activation of JAK2. In this regard, the effect of leptin in JAK2 deficient mice was of particular interest. The study would, however, require conditional JAK2 deletion, as germline deletion of Jak2 is embryonically lethal due to impaired hematopoiesis (Park et al. 2013).

JAK2 is further involved in the signaling of growth hormone (Brooks and Waters 2010; Xia et al. 2002; Yang et al. 2010), which up-regulates ENaC (Kamenicky et al. 2008). The effect of growth hormone may, however, be mainly due to up-regulation of signal transducer and activator of transcription 5 (STAT5), a transcription factor binding to a response element located in the αENaC promoter (Kamenicky et al. 2008). Clearly, additional experimental effort is needed to define the functional role and mechanisms of JAK2-sensitive ENaC activity and its contribution to the regulation of Na^+ transport by leptin and growth hormone.

Most recent obervations revealed energy sensitivity of JAK2 (Bhavsar et al. 2013). The kinase is activated by energy depletion. The inhibition of ENaC decreases Na^+ entry, and thus the requirement for energy consuming Na^+ extrusion by the Na^+/K^+ ATPase. Thus, inhibition of ENaC by JAK2 may be part of the cellular strategy to survive transient energy depletion.

In conclusion, the present paper reveals that JAK2 downregulates the epithelial Na^+ channel ENaC, an effect most likely affecting transpithelial salt transport.

Acknowledgments The authors acknowledge the meticulous preparation of the manuscript by Lejla Subasic and technical support by Elfriede Faber. This study was supported by the Deutsche Forschungsgemeinschaft.

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