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Effect of TGFβ on Na^+/K^+ ATPase activity in megakaryocytes

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ABSTRACT

The Na^+/K^+ ATPase generates the Na^+ and K^+ concentration gradients across the plasma membrane and is thus essential for cellular electrolyte homeostasis, cell membrane potential and cell volume maintenance. A powerful regulator of Na^+/K^+ ATPase is the serum- and glucocorticoid-inducible kinase 1 (SGK1). The most powerful known regulator of SGK1 expression is TGFß1, which is pivotal in the regulation of megakaryocyte maturation and platelet formation. Signaling involved in the upregulation of SGK1 by TGFß1 includes p38 mitogen activated protein (MAP) kinase. SGK1 in turn phosphorylates the IκB kinase (IKKα/β), which phosphorylates the inhibitor protein IκBα thus triggering nuclear translocation of nuclear factor kappa B (NF-κB). The present study explored whether TGFβ influences Na^+/K^+ ATPase activity in megakaryocytes, and if so, whether the effect of TGß1 requires p38 MAP kinase, SGK1 and/or NF-κB. To this end, murine megakaryocytes were treated with TGFß1 and Na^+/K^+ ATPase activity determined from K^+ induced current utilizing whole cell patch clamp. The pump current (I_{pump}) was determined in the absence and presence of Na^+/K^+ ATPase inhibitor ouabain (100 μM). TGFß1 (60 ng/ml) was added in the absence or presence of p38 MAP kinase inhibitor skepinone-L (1 μM), SGK1 inhibitor EMD638683 (50 μM) or NF-κB inhibitor wogonin (50 nM). As a result, the I_{pump} was significantly increased by pretreatment of the megakaryocytes with TGFß1, an effect reaching statistical significance within 16 and 24 h and virtually abrogated in the presence of skepinone-L, EMD638683 or wogonin. In conclusion, TGFß1 is a powerful regulator of megakaryocytic Na^+/K^+ ATPase activity. Signaling mediating the effect of TGFß1 on Na^+/K^+ ATPase activity involves p38 MAP kinase, SGK1 and NF-κB.

1. Introduction

Platelets accomplish primary hemostasis following vascular injury and participate in the development of thrombosis and thrombotic vascular occlusion [1,2]. Platelet activation is followed by degranulation, triggering of coagulation, phosphatidylserine exposure, aggregation and thrombus formation [2,3]. All those events require an increase of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$), which is accomplished by Ca^{2+} release from intracellular stores and subsequent triggering of store operated calcium entry (SOCE) [4,5].

PI3K and phosphoinositide-dependent kinase PDK1 are both potent regulators of platelet function triggering glycogen synthase kinase-3 (GSK3) β-mediated integrin $\alpha_{IIb}\beta_3$ activation and consecutive outside-in signaling following thrombin-induced platelet activation [6–11]. PI3K dependent kinases include the serum- and glucocorticoid-inducible kinase 1 (SGK1) [12–14], a powerful stimulator of several ion channels including the Ca^{2+} channel Orai1 [11,15]. SGK1 phosphorylates and thus activates the IκB kinase (IKKα/β), which in turn phosphorylates the inhibitor protein IκBα thus leading to nuclear translocation of nuclear factor NF-κB and NF-κB-dependent Orai1 expression [15,16]. Stimulators of SGK1 transcription include transforming growth factor TGFβ [17], a powerful inhibitor of megakaryocyte maturation [18]. TGFß1 upregulates SGK1 transcription through activation of p38 MAP kinase [19].

As shown in other cell types, SGK1 sensitive transport processes include the Na^+/K^+ ATPase [20], the most prominent member of the P-type adenosine triphosphatase (ATPase) family [21]. In one transport cycle, the Na^+/K^+ ATPase pumps three Na^+ ions out of and two K^+ ions into the cell and consumes one molecule of ATP

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[22,23]. The Na^+/K^+ ATPase thus generates an outward current, establishes the Na^+ and K^+ concentration gradients across the plasma membrane [22,23] and is thus pivotal for maintenance of cell membrane potential, cellular electrolyte homeostasis and cell volume regulation.

The present study explored, whether TGFß1 influences Na^+/K^+ ATPase activity in megakaryocytes. As a matter of fact, TGFß1 is a powerful stimulator of Na^+/K^+ ATPase activity. Further experiments explored whether regulation of Na^+/K^+ ATPase activity by TGFß1 involves p38 MAP kinase, SGK1 and/or NF-κB.

2. Materials and methods

2.1. Mice

All animal experiments were conducted according to the German law for the welfare of animals and were approved by local authorities (Regierungspräsidium Tübingen). Male and female mice were studied at the age of 8–12 weeks. The mice had free access to water ad libitum and to a standard mouse diet (C1310 Altromin, Heidenau, Germany).

2.2. Inhibitors

The specific p38 MAP kinase inhibitor skepinone-L was generated by S. Laufer (University of Tübingen, Germany) as described previously [24], the SGK1 inhibitor [25–27] was kindly provided by Merck Darmstadt, the NF-κB inhibitor wogonin [24] was purchased from Sigma (Taufkirchen, Germany).

2.3. Isolation and culture of murine megakaryocytes

Murine megakaryocytes were isolated as described previously [4,15]. For the isolation of murine megakaryocytes, bone marrow cells were harvested by flushing the femurs and tibias with phosphate-buffered saline (Life Technologies, Darmstadt, Germany) as described previously [28]. The obtained cells were separated over Percoll (GE Healthcare, Little Chalfont, UK) and cultured in specific growth medium (MethoCult, Stemcell, Grenoble, France) containing 10% fetal bovine serum (Life Technologies), 20% IMDM with Glutamax (Life Technologies), and 50 ng/ml thrombopoietin (Invitrogen, Carlsbad, CA, USA) as described previously [29]. After 7 d, differentiation into megakaryocytes was tested by microscopy, as well as glycoprotein Ibα (GPIbα) staining and Western blotting.

2.4. Patch clamp

Ouabain-sensitive K^+-induced currents (I_{pump}) reflecting Na^+/K^+-ATPase activity were determined by whole cell patch clamp recording prior to and following a 6, 16, 24 h treatment with TGFß1 (20, 40, 60 ng/ml) in the presence or absence of inhibitors.

Patch clamp experiments were performed at room temperature in voltage-clamp, fast whole cell mode [30–32]. Cells were continuously superfused through a flow system inserted into the dish. The bath was grounded via a bridge filled with the external solution (see below). Borosilicate glass pipettes (2- to 4-MΩ resistance; Harvard Apparatus, 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) and analyzed with Pulse software (Heka) and an ITC-16 Interface (Instrutech, Port Washington, NY). Currents were recorded at an acquisition frequency of 10 kHz and 3 kHz low-pass filtered.

To measure Na^+/K^+ ATPase activity, ouabain (100 μM) sensitive K^+-induced outward currents were recorded. The pipette solution used contained (in mM): 30 NaCl, 20 KCl, 70 CsCl, 5 $MgCl_2$, 5 HEPES, 5 Na_2ATP and 5 ethylene glycol tetraacetic acid (EGTA). The external solution contained (in mM) 60 NaCl, 80 TEA-Cl, 1 $MgCl_2$, 2.5 $CaCl_2$, 5 $NiCl_2$ 5 glucose, 10 HEPES (pH 7.4, CsOH), and 0.5 EGTA. Na^+/K^+ ATPase currents were elicited by switching to a bath solution that contained 60 NaCl, 80 TEA-Cl, 5 KCl, 1 $MgCl_2$, 2.5 $CaCl_2$, 5 $NiCl_2$ 5 glucose, 10 HEPES (pH 7.4, CsOH). The currents were measured at −40 mV.

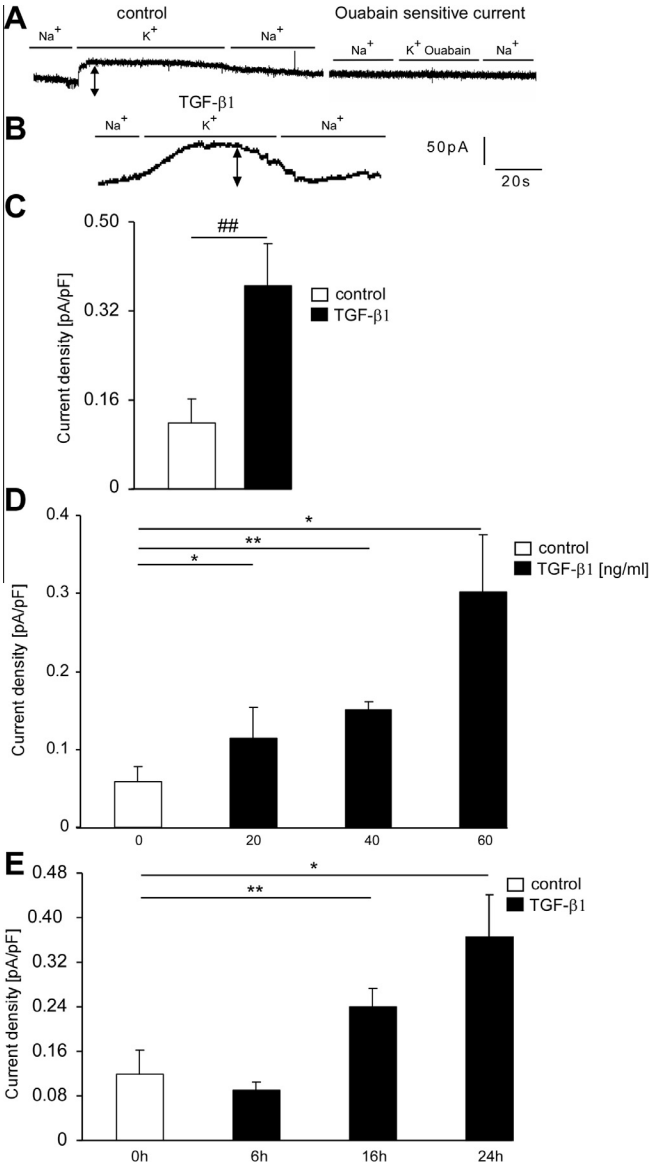

Fig. 1. Effect of TGFß1 on Na^+/K^+-ATPase currents in megakaryocytes. (A, B) Original whole cell tracings recorded at −40 mV in untreated (A) and TGFß1 (60 ng/ml for 24 h) treated (B) megakaryocytes in the absence (Na^+) and in the presence of 5 mM K^+ (K^+) in the bath solution. In untreated megakaryocytes megakaryocytes (A) K^+ was added either in the absence (left) or in the presence (right) of ouabain (100 μM). (C) Mean whole-cell current at −40 mV normalized to cell capacitance (current density ± SEM, $n = 5$) following addition of 5 mM K^+. The megakaryocytes were not treated (white bars) or treated (black bars) for 24 h with TGFß1. (D, E) Mean whole-cell current at −40 mV normalized to cell capacitance (current density ± SEM, $n = 4–12$) following addition of 5 mM K^+ in untreated megakaryocytes (white bars) or in megakaryocytes treated (D) for 24 h with the indicated TGFß1 concentrations (20–60 ng/ml) or (E) with 60 ng/ml TGFß1 for the indicated time (6–24 h) periods. $^*(p < 0.05)$ and $^{**}(p < 0.01)$ indicate statistically significant difference (unpaired t-test or ANOVA).

2.5. Statistical analysis

Data are provided as means ± SEM, n represents the number of experiments. All data were tested for significance using paired or unpaired Student t-test and one-way ANOVA with Dunnett's post-hoc test. Results with $p < 0.05$ were considered statistically significant.

3. Results

Whole cell patch clamp was employed to elucidate the effect on Na^+/K^+ ATPase activity. Electrogenic transport by the Na^+/K^+ ATPase was triggered by addition of K^+ to the extracellular solution leading to electrogenic extrusion of 3 Na^+ in exchange for 2 K^+. As illustrated in Fig. 1, the addition of K^+ triggered a current (I_{pump}) in megakaryocytes, which was fully reversed by the Na^+/K^+ ATPase inhibitor ouabain (100 μM). I_{pump} was significantly increased by a 24 h pretreatment of the megakaryocytes with TGFß1 (20, 40 and 60 ng/ml). Following application of 60 ng/ml, the effect reached statistical significance within 16 h.

In order to elucidate the involvement of p38 MAP kinase in TGFß1 sensitive regulation of Na^+/K^+-ATPase activity the experiments were performed in the absence or presence of the p38 MAP kinase inhibitor skepinone-L [24,33]. As shown in Fig. 2, the effect of TGFß1 (60 ng/ml, 24 h) was fully reversed by treatment of the megakaryocytes in the presence of skepinone-L (1 μM, 24 h).

As p38 MAP kinase upregulates the expression of SGK1, which is in turn known to up-regulate Na^+/K^+ ATPase activity, additional experiments addressed the role of SGK1. To this end, the K^+ induced current was determined in megakaryocytes treated with TGFß1 (60 ng/ml, 24 h in the absence and presence of SGK1 inhibitor EMD638683 (50 μM, 24 h) [25–27]. As illustrated in Fig. 3, the effect of TGFß1 was fully reversed by treatment of the megakaryocytes in the presence of EMD638683 (50 μM, 24 h).

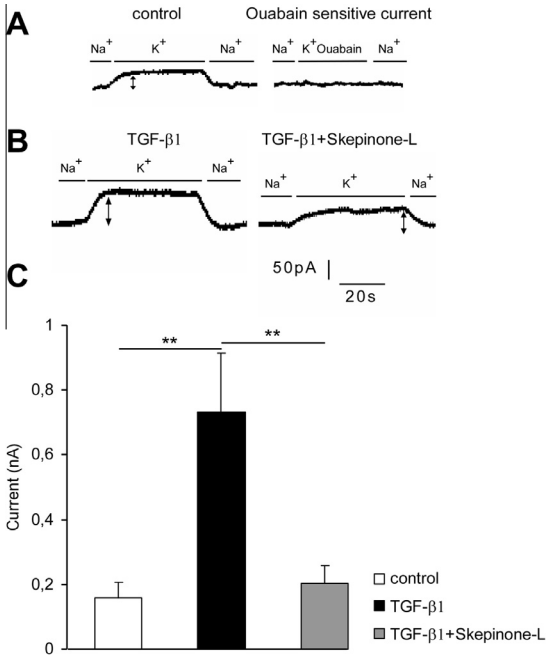

Fig. 2. Effect of TGFß1 on Na^+/K^+-ATPase currents in megakaryocytes in the presence of p38 MAP kinase inhibitor skepinone-L. (A, B) Original whole cell tracings recorded at −40 mV) in untreated (A) and TGFß1 (60 ng/ml for 24 h) treated (B) megakaryocytes in the absence (Na^+) and in the presence of 5 mM K^+ (K^+) in the bath solution. (A) In untreated megakaryocytes K^+ was added either in the absence (left) or in the presence (right) of ouabain (100 μM). (B) TGFß1 (60 ng/ml for 24 h) was added either without (left) or with (right) p38 MAP kinase inhibitor skepinone-L (1 μM). (C) Mean whole-cell current at −40 mV normalized to cell capacitance (current density ± SEM, n = 6–7) following addition of 5 mM K^+. The megakaryocytes were not treated (white bars) or treated with 60 ng/ml for 24 h with TGFß1 in the absence (black bar) or presence (gray bar) of skepinone-L (1 μM, 24 h). $^{**}(p < 0.01)$ indicates statistically significant difference (ANOVA).

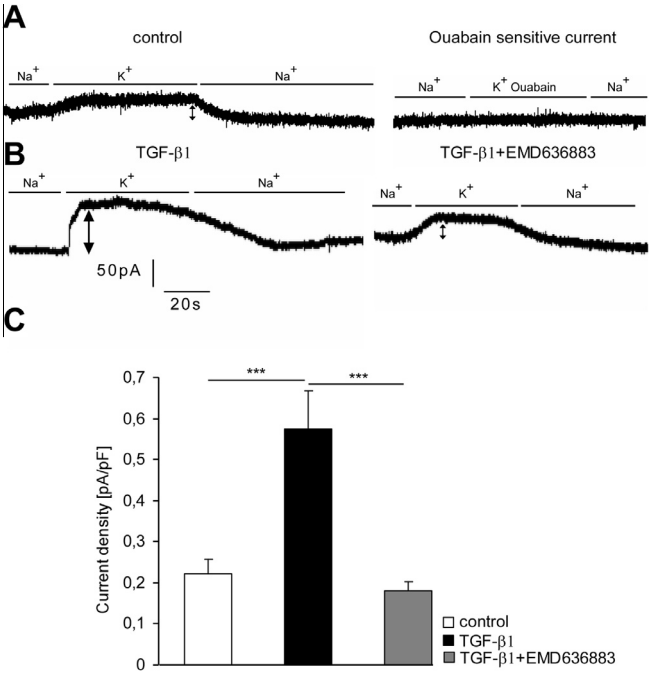

Fig. 3. Effect of TGFß1 on Na^+/K^+-ATPase currents in megakaryocytes in the presence of SGK1 inhibitor EMD638683. (A, B) Original whole cell tracings recorded at −40 mV in untreated (A) and TGFß1 (60 ng/ml for 24 h) treated (B) megakaryocytes in the absence (Na^+) and in the presence of 5 mM K^+ (K^+) in the bath solution. (A) In untreated megakaryocytes K^+ was added either in the absence (left) or in the presence (right) of ouabain (100 μM). (B) TGFß1 (60 ng/ml for 24 h) was added either without (left) or with (right) SGK1 inhibitor EMD638683 (50 μM, 24 h). (C) Mean whole-cell current at −40 mV normalized to cell capacitance (current density ± SEM, n = 8–12) following addition of 5 mM K^+. The megakaryocytes were not treated (white bars) or treated with 60 ng/ml for 24 h with TGFß1 in the absence (black bar) or presence (gray bar) of 50 μM EMD638683 for 24 h. $^{***}(p < 0.001)$ indicates statistically significant difference (ANOVA).

SGK1 is in part effective by up-regulating the transcription factor NF-κB. In order to elucidate the involvement of NF-κB in TGFß1 sensitive regulation of Na^+/K^+-ATPase activity, the experiments were repeated in the absence and presence of NF-κB inhibitor wogonin [24]. As illustrated in Fig. 4, the effect of TGFß1 (60 ng/ml, 24 h) was fully reversed by treatment of the megakaryocytes in the presence of wogonin (50 nM, 24 h).

4. Discussion

The present observations reveal a completely novel effect of TGFß1 in megakaryocytes, i.e. upregulation of Na^+/K^+-ATPase activity. Within 16 h exposure of megakaryocytes to TGFß1 leads to marked upregulation of K^+ induced pump current. The effect is slow and not yet apparent 6 h after TGFß1 treatment. The effect is apparently dependent on the activity of p38 MAP kinase, SGK1 and NF-κB. TGFß1 signaling involves p38 MAP kinase [34], which in turn is known to upregulate SGK1 expression [35,36]. As a matter of fact, TGFß1 is a most powerful stimulator of SGK1 transcription [17], an effect requiring p38 MAP kinase [19]. SGK1 activates NF-κB by phosphorylation and thus activation of the IκB kinase (IKKα/β), a kinase phosphorylating and thus downregulating the NF-κB inhibiting protein IκBα [15,16].

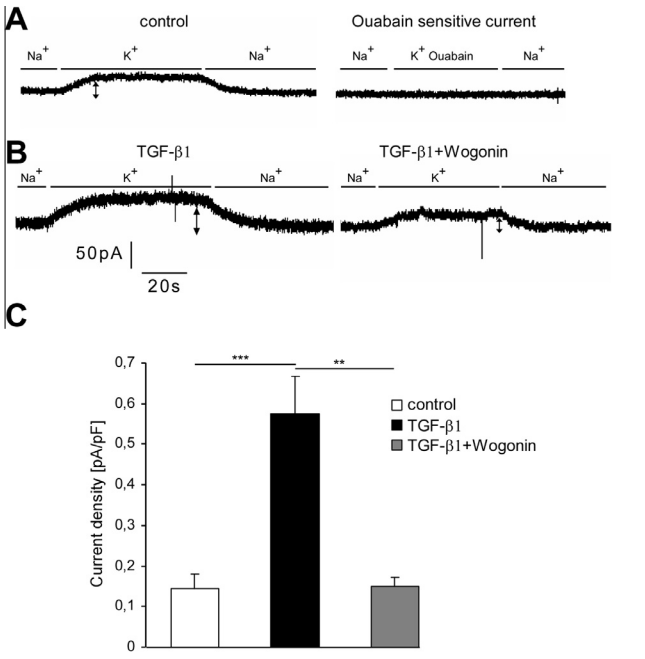

Fig. 4. Effect of TGFß1 on Na^+/K^+-ATPase currents in megakaryocytes in the presence of NF-κB inhibitor wogonin. (A, B) Original whole cell tracings recorded at −40 mV in untreated (A) and TGFß1 (60 ng/ml for 24 h) treated (B) megakaryocytes in the absence (Na^+) and in the presence of 5 mM K^+ (K^+) in the bath solution. (A) In untreated megakaryocytes K^+ was added either in the absence (left) or in the presence (right) of ouabain (100 μM). (B) TGFß1 (60 ng/ml for 24 h) was added either without (left) or with (right) NF-κB inhibitor wogonin (50 nM) (B). (C) Mean whole-cell current at −40 mV normalized to cell capacitance (current density ± SEM, $n = 5–15$) following addition of 5 mM K^+. The megakaryocytes were not treated (white bars) or treated with 60 ng/ml for 24 h with TGFß1 in the absence (black bar) or presence (gray bar) of 50 nM wogonin. $^{**}(p < 0.01)$ and $^{***}(p < 0.001)$ indicate statistically significant difference (ANOVA).

Activation of p38 MAP kinase, SGK1 and/or NF-κB as well as upregulation of Na^+/K^+-ATPase activity may contribute to the machinery underlying the known powerful inhibition of megakaryocyte maturation by TGFß1 [18]. It is noteworthy that inhibition of Na^+/K^+-ATPase activity with ouabain stimulates the expression of TGFß1 [37]. The upregulation of Na^+/K^+ ATPase activity by TGFß1 may thus be part of a negative feedback loop.

The Na^+/K^+-ATPase activity impacts on cellular energy metabolism. In resting cells Na^+/K^+ ATPase activity may consume come 30% of cellular ATP consumption [38–41]. Enhanced Na^+/K^+-ATPase activity thus increases energy consumption and may thus aggravate energy shortage [42].

Stimulation of the Na^+/K^+ ATPase is further expected to increase the Na^+ and K^+ concentration gradients across the plasma membrane [22,23]. The Na^+ concentration gradient drives a variety of transport processes including the Na^+/Ca^{2+} exchangers [43–45]. The K^+ ions accumulated by the Na^+/K^+ ATPase exit through K^+-channels and thus establish the potential difference across the cell membrane [46]. Na^+/K^+-ATPase activity thus impacts on K^+ conductance thus modifying the cell membrane potential and the driving forces for electrogenic transport processes [47–49]. Collectively, hyperpolarization and decrease of cytosolic Na^+ concentration are expected to foster extrusion of Ca^{2+} by the Na^+/Ca^{2+} exchangers and thus to suppress Ca^{2+} signaling.

Na^+/K^+ ATPase activity is apparently decreased in platelets from patients affected by acute stroke [50]. In those patients the platelet Na^+/K^+ ATPase activity is negatively correlated with the National Institute of Health Stroke Scale [50]. Thus, enhanced platelet Na^+/K^+ ATPase activity may be protective against untoward platelet activation and vascular occlusion.

In conclusion, TGFß1 upregulates the megakaryocytic Na^+/K^+-ATPase, an effect mediated by a signaling cascade involving p38 MAP kinase, SGK1 and NF-κB.

Authorship contributions

Z.H. and E.S. performed experiments and analyzed data. E.S., S.L. and O.B. analyzed data and contributed to writing of the manuscript. M.G. and F.L. designed the study. F.L. drafted the manuscript. All authors corrected and approved the manuscript.

Conflict of interest

The authors state that they have no conflict of interest.

Acknowledgments

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