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Original Paper

P38 Kinase, SGK1 and NF-κB Dependent Up-Regulation of Na^+/Ca^{2+} Exchanger Expression and Activity Following TGFß1 Treatment of Megakaryocytes

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Key Words
NCX1 • NCKX1 • NCKX2 • NCKX5 • Calcium • p38 kinase • SGK1 • NF-κB

Abstract
Background: TGFβ1, a decisive regulator of megakaryocyte maturation and platelet formation, has previously been shown to up-regulate both, store operated Ca^{2+} entry (SOCE) and Ca^{2+} extrusion by Na^+/Ca^{2+} exchange. The growth factor thus augments the increase of cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$) following release of Ca^{2+} from intracellular stores and accelerates the subsequent decline of $[Ca^{2+}]_i$. The effect on SOCE is dependent on a signaling cascade including p38 kinase, serum & glucocorticoid inducible kinase SGK1, and nuclear factor NFκB. The specific Na^+/Ca^{2+} exchanger isoforms involved and the signalling regulating the Na^+/Ca^{2+} exchangers remained, however elusive. The present study explored, whether TGFβ1 influences the expression and function of K^+ insensitive (NCX) and K^+ sensitive (NCKX) Na^+/Ca^{2+} exchangers, and aimed to shed light on the signalling involved. ***Methods:*** In human megakaryocytic cells (MEG01) RT-PCR was performed to quantify NCX/NCKX isoform transcript levels, $[Ca^{2+}]_i$ was determined by Fura-2 fluorescence, and Na^+/Ca^{2+} exchanger activity was estimated from the increase of $[Ca^{2+}]_i$ following switch from an extracellular solution with 130 or 90 mM Na^+ and 0 mM Ca^{2+} to an extracellular solution with 0 Na^+ and 2 mM Ca^{2+}. K^+ concentration was 0 mM for analysis of NCX and 40 mM for analysis of NCKX. ***Results:*** TGFβ1 (60 ng/ml, 24 h) significantly increased the transcript levels of NCX1, NCKX1, NCKX2 and NCKX5. Moreover, TGFβ1 (60 ng/ml, 24 h) significantly increased the activity of both, NCX and NCKX. The effect of TGFβ1 on NCX and NCKX transcript levels and

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activity was significantly blunted by p38 kinase inhibitor Skepinone-L (1 µM), the effect on NCX and NCKX activity further by SGK1 inhibitor GSK-650394 (10 µM) and NFκB inhibitor Wogonin (100 µM). ***Conclusions:*** TGFβ1 markedly up-regulates transcription of NCX1, NCKX1, NCKX2, and NCKX5 and thus Na^+/Ca^{2+} exchanger activity, an effect requiring p38 kinase, SGK1 and NFκB.

Introduction

Platelets are decisive for the accomplishment of primary haemostasis and are key players in the development of acute thrombosis and thrombotic vascular occlusion [1]. Activation of platelets is followed by degranulation, exposure of phosphatidylserine, aggregation and thrombus formation [2]. All those functions are triggered by an increase of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) [3, 4]. Ca^{2+} is increased by stimulation of Ca^{2+} release from intracellular stores and subsequent activation of store operated calcium entry (SOCE) [5]. SOCE is accomplished by the Ca^{2+} permeable pore forming calcium release-activated channel (CRAC) moiety Orai1 (CRACM1) and its regulator stromal interaction molecule 1 (STIM1), which senses the Ca^{2+} content of the intracellular Ca^{2+} stores [6-8].

Signaling regulating Orai1 abundance in platelets include phosphoinositide 3-kinase (PI3K) [9-12] which participates in activation of Serum- and Glucocorticoid-inducible Kinase 1 (SGK1) [13, 14], a powerful stimulator of Orai1 expression [15]. SGK1 is in part effective by phosphorylation and thus activation of IκB kinase (IKKα/β), which in turn phosphorylates the inhibitor protein IκBα resulting in nuclear translocation of nuclear factor NFκB [15, 16].

SGK1 is strongly up-regulated by transforming growth factor TGFβ [17], an effect dependent on activation of the p38 kinase [18]. TGFß1, an inhibitor of megakaryocyte maturation released from platelets following increase of cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$) [19], is thus a powerful stimulator of Orai1 and SOCE in megakaryocytes, an effect dependent on p38 kinase, SGK1 and NF-κB [20].

TGFß1 further up-regulates Na^+/Ca^{2+} exchange [21], which accomplishes extrusion of Ca^{2+} thus decreasing $[Ca^{2+}]_i$ and contributing to duration and amplitude Ca^{2+} signals [22-25]. Ca^{2+} extrusion by Na^+/Ca^{2+} exchangers is driven by the low cytosolic Na^+ concentration and the potential difference across the cell membrane [22]. Ca^{2+} transport of the carrier may be reversed by decrease of the inwardly directed Na^+ gradient across the cell membrane and/or cell membrane depolarization, which may thus lead to Ca^{2+} entry through the carrier [22, 26]. The family of Na^+/Ca^{2+} exchangers includes 6 K^+-dependent (NCKX) and 3 K^+-independent (NCX) Na^+/Ca^{2+} exchanger isoforms [27-29]. The NCKX isoforms exchange one K^+ ion and one Ca^{2+} ion for four Na^+ ions and the NCX isoforms exchange three Na^+ ions for one Ca^{2+} ion [30].

The previous study did not address the NCX and NCKX isoforms regulated by TGFß1, nor the signaling involved. The present study thus explored whether TGFß1 increases the transcript levels of NCX and NCKX isoforms and elucidated the role of p38 kinase, SGK1 and NF-κB.

Materials and Methods

Cell culture of megakaryocytes

Human megakaryocytic cells (MEG01) from ATCC (American Type Culture Collection) were cultured in 10% FBS (fetal bovine serum) and 1% Penicillin/ Streptomycin containing RPMI 1640 (Roswell Park Memorial Institute) medium (Gibco ThermoFischer Scientific) in humidified atmosphere at 37°C and 5% CO_2. Where indicated, TGFß1 (60 ng/ml, Sigma, Taufkirchen, Germany), p38 kinase inhibitor Skepinone-L [31] (1 µM, Merck), SGK1 inhibitor GSK-650394 (10 µM, Tocris), or NFκB inhibitor Wogonin (100 µM, Sigma) were added to the medium.

q-Real-time PCR

Total RNA was extracted in TriFast (Peqlab, Erlangen, Germany) according to the manufacturer's instructions. 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, Hilden, Germany) according to the manufacturer's protocol. Cycling conditions were as follows: initial denaturation at 95°C for 5 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 NCX1:
fw: ACAAGAGGTATCGAGCTGGC
rev: ATGCCATTTCTCGCCTAGC
for NCKX1:
fw: TCCACGCAGAAGATGGTG
rev GTGATGGAGGGGATAGCG
for NCKX2:
fw: GAGACAGATACACAGAGCACAGG
rev: GAGAATAGTACAGATCACGCCC
for NCKX5:
fw: CTCCATCGGAGTTCC
rev: CTTCCTACCCTCCCTGGAA
for GAPDH:
fw: TGAGTACGTCGTGGAGTCCAC
rev: GTGCTAAGCAGTTGGTGGTG

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) and all experiments were done in duplicate. The housekeeping gene GAPDH (Glyceraldehyd-3-phosphate-Dehydrogenase) was amplified to standardize the amount of sample RNA. Relative quantification of gene expression was achieved using the ΔCT method as described earlier [32, 33].

Ca^{2+} measurements

Fura-2 fluorescence was utilized to determine intracellular Ca^{2+} activity [34]. Cells were loaded with Fura-2/AM (2 µM, Invitrogen, Goettingen, Germany) for 20-60 min at 37°C. Cells were excited alternatively at 340 nm and 380 nm through an objective (Fluor 40×/1.30 oil) built in a fluorescence 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) [35].

Na^+/Ca^{2+} exchanger activity was estimated from the changes in cytosolic Ca^{2+} activity upon replacement of extracellular Na^+ by N-methyl-d-glucamine (NMDG). The standard Na^+ containing solution was composed of (in mM): 130 NaCl, 0 KCl, 2 $MgCl_2$, 10 HEPES, 5 glucose, pH 7.4 and the Na^+-free solution of (in mM): 90 NMDG, 0 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, 5 glucose, pH 7.4. For determination of NCKX activity the Na^+ containing solution was composed of (in mM): 130 NaCl, 40 KCl, 20 TAE^+, 2 $MgSO_4$, 10 HEPES, 5 glucose, pH 7.4 and the Na^+-free solution of (in mM): 90 NMDG, 40 KCl, 20 TAE^+, 2 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, 5 glucose, pH 7.4. For quantification of Ca^{2+} entry, the slope (delta ratio/s) and peak (delta ratio) were calculated following removal of Na^+.

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 Dunnets post-hoc test. Results with $^{\$}$/*($p<0.05$), $^{\$\$}$/**($p<0.01$) or §§§/***($p<0.001$) were considered statistically significant.

Results

The present study addressed the mechanisms involved in the stimulation of Na^+/Ca^{2+} exchange by TGFß1. In a first series of experiments, RT-PCR was employed in order to define the NCX and NCKX isoforms involved. As illustrated in Fig. 1, a 24 hours treatment with 60

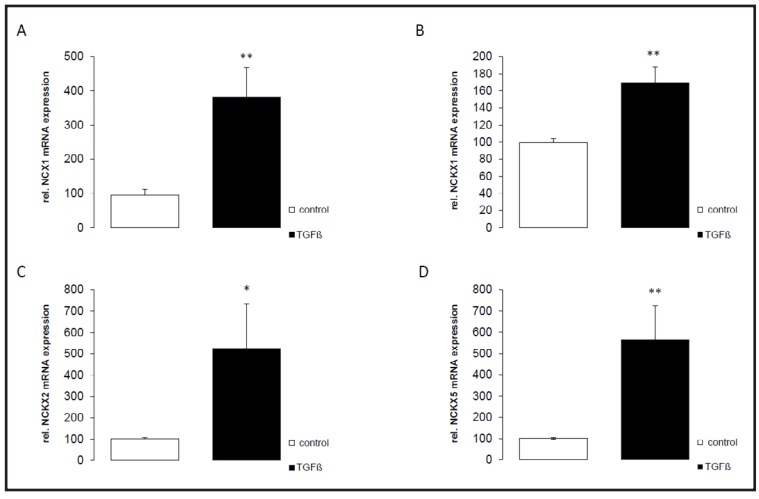

Fig. 1. TGFß1 sensitive expression of NCX1, NCKX1, NCKX2, and NCKX5 isoforms in megakaryocytes. A-D: Arithmetic means (± SEM, n = 5-11 preparations) of (A) NCX1, (B) NCKX1, (C) NCKX2, and (D) NCKX5 over GAPDH transcript levels in megakaryocytes without (white bars) and with (black bars) prior TGFß1 treatment (60 ng/ml, 24 hours). *(p<0.05), ** (p<0.01) indicates statistically significant difference from absence of TGFß1 (student´s t-test).

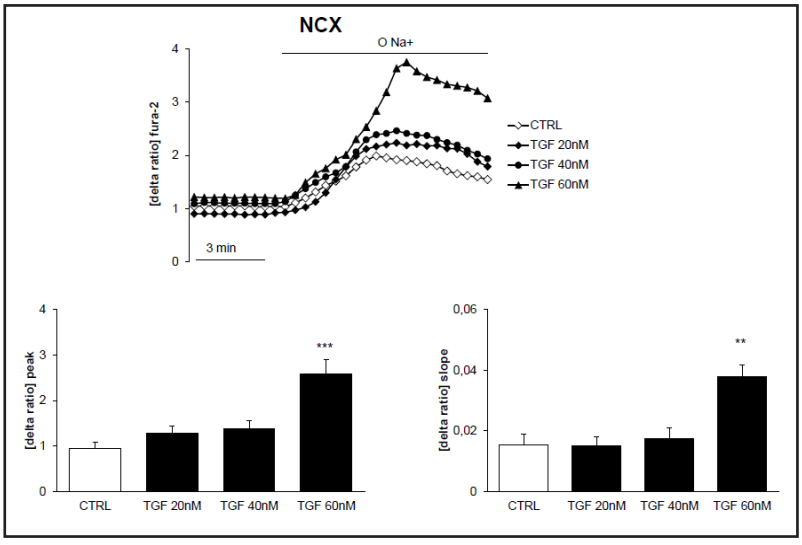

Fig. 2. TGFß1 sensitive NCX mediated Ca^{2+} entry in megakaryocytes. A. Representative original tracings showing intracellular Ca^{2+} concentrations in Fura-2/AM loaded megakaryocytes without (open diamonds) and with prior 24 h treatment with 20 ng/ml TGFß1 (closed diamonds), 40 ng/ml TGFß1 (closed circles) or 60 ng/ml TGFß1 (closed triangles) prior to and following removal of external Na^+ (0 Na^+) and adding 2 mM Ca^{2+} at 0 mM K^+. B,C. Arithmetic means (± SEM, n = 46 - 59 cells) of the peak (B) and slope (C) of the change in intracellular Ca^{2+} concentrations following removal of external Na^+ (0 Na^+) and adding 2 mM Ca^{2+} at 0 mM K^+ in megakaryocytes without (white bars) and with (black bars) prior TGFß1 treatment. ** (p<0.01), *** (p<0.001) indicate statistically significant difference from untreated megakaryocytes (ANOVA).

ng/ml TGFß1 was followed by a significant increase of the transcript levels encoding NCX1, NCKX1, NCKX2, and NCKX5. Thus, TGFß1 stimulates the transcription of several NCX and NCKX isoforms.

In order to quantify Na^+/Ca^{2+} exchanger activity, cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$) was determined utilizing Fura-2 fluorescence. Na^+/Ca^{2+} exchanger activity was estimated from the increase of $[Ca^{2+}]_i$ following reversal of the Na^+/Ca^{2+} exchanger by replacing extracellular Na^+ with $NMDG^+$ and simultaneous addition of extracellular Ca^{2+}. In a first series of experiments no extracellular K^+ was added to specifically observe NCX activity. As shown in Fig. 2, pre-treatment with 60 ng/ml but not pre-treatment with 20 ng/ml or 40 ng/ml TGFß1 was followed by a marked and statistically significant increase of K^+ independent Na^+/Ca^{2+} exchanger activity. In a second series of experiments 40 mM extracellular K^+ was added to observe NCKX activity. As shown in Fig. 3, again pre-treatment with 60 ng/ml but not pre-treatment with 20 ng/ml or 40 ng/ml TGFß1 was followed by a marked and statistically significant increase of K^+ dependent Na^+/Ca^{2+} exchanger activity.

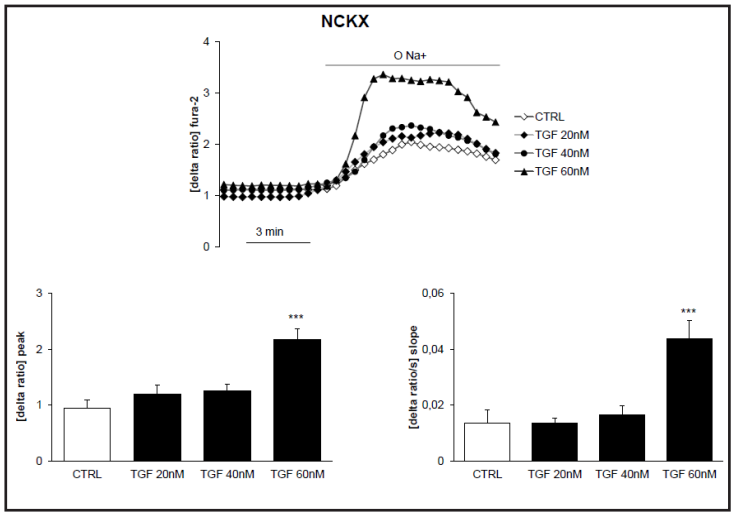

Fig. 3. TGFß1 sensitive NCKX mediated Ca^{2+} entry in megakaryocytes. A. Representative original tracings showing intracellular Ca^{2+} concentrations in Fura-2/AM loaded megakaryocytes without (open diamonds) and with prior 24 h treatment with 20 ng/ml TGFß1 (closed diamonds), 40 ng/ml TGFß1 (closed circles) or 60 ng/ml TGFß1 (closed triangles) prior to and following removal of external Na^+ (0 Na^+) and adding 2 mM Ca^{2+} at 40 mM K^+. B,C. Arithmetic means (± SEM, n = 46 - 59 cells) of the peak (B) and slope (C) of the change in intracellular Ca^{2+} concentrations following removal of external Na^+ (0 Na^+) and adding 2 mM Ca^{2+} at 40 mM K^+ in megakaryocytes without (white bars) and with (black bars) prior TGFß1 treatment. *** (p<0.001) indicate statistically significant difference from untreated megakaryocytes (ANOVA).

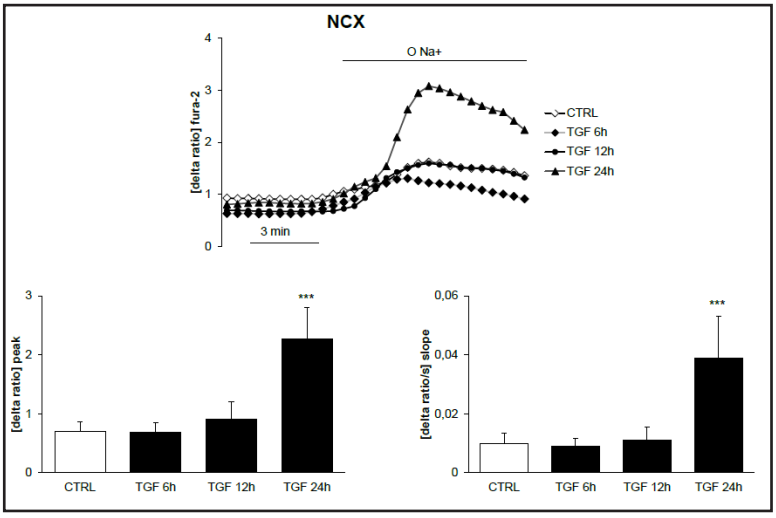

Fig. 4. Time course of TGFß1-induced increase of NCX mediated Ca^{2+} entry in megakaryocytes. A. Representative original tracings showing intracellular Ca^{2+} concentrations in Fura-2/AM loaded megakaryocytes without (open diamonds) and with prior treatment with 60 ng/ml TGFß1 for 6 hours (closed diamonds), 12 hours (closed circles) or 24 hours (closed triangles) prior to and following removal of external Na^+ (0 Na^+) and adding 2 mM Ca^{2+} at 0 mM K^+. B,C. Arithmetic means (± SEM, n = 40 - 52 cells) of the peak (B) and slope (C) of the change in intracellular Ca^{2+} concentrations following removal of external Na^+ (0 Na^+) and adding 2 mM Ca^{2+} at 0 mM K^+ in megakaryocytes without (white bars) and with (black bars) prior TGFß1 (60 nM) treatment. *** (p<0.001) indicate statistically significant difference from untreated megakaryocytes (ANOVA).

In a further series of experiments the time course of the TGFß1 effect was elucidated. As shown in Fig. 4, pre-treatment with 60 ng/ml TGFß1 for 24 hours, but not for 6 hours or 12 hours was followed by a marked and statistically significant increase of K^+ independent Na^+/Ca^{2+} exchanger activity. As illustrated in Fig. 5, pre-treatment with 60 ng/ml TGFß1 for 24 hours, but not for 6 hours or 12 hours was followed by a marked and statistically significant increase of K^+ independent Na^+/Ca^{2+} exchanger activity

The involvement of the p38 kinase in the up-regulation of Na^+/Ca^{2+} exchanger transcription and activity following TGFß1 treatment of megakaryocytes was tested by application of the p38 kinase inhibitor Skepinone-L (1 µM). As shown in Fig. 6, a 24 hours treatment with 60 ng/ml TGFß1 again significantly increased the transcript levels encoding NCX1, NCKX1, NCKX2, and NCKX5. The effect of TGFß1 on the transcript levels was abrogated in the presence of Skepinone-L. The alterations of the transcript levels were paralleled by the respective alterations of Na^+/Ca^{2+} exchanger activity. As illustrated in Fig. 7, the up-regulation of

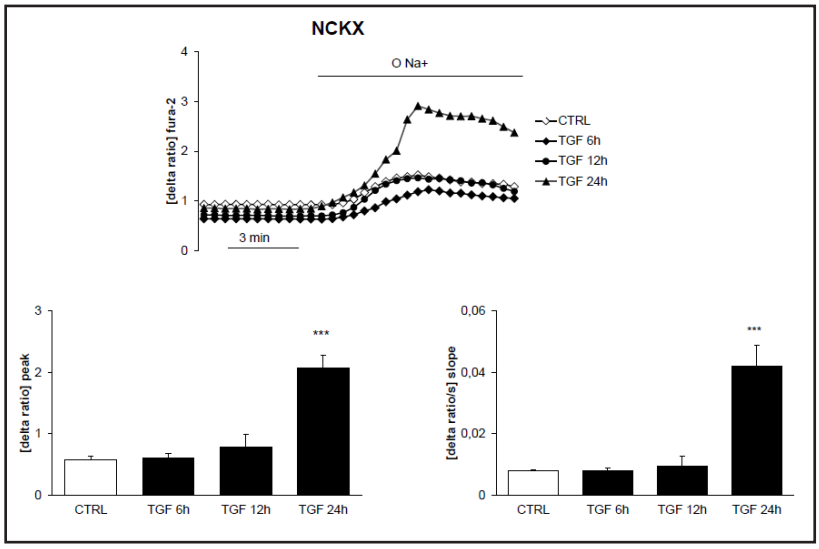

Fig. 5. Time course of TGFß1-induced increase of NCKX mediated Ca^{2+} entry in megakaryocytes. A. Representative original tracings showing intracellular Ca^{2+} concentrations in Fura-2/AM loaded megakaryocytes without (open diamonds) and with prior treatment with 60 ng/ml TGFß1 for 6 hours (closed diamonds), 12 hours (closed circles) or 24 hours (closed triangles) prior to and following removal of external Na^+ (0 Na^+) and adding 2 mM Ca^{2+} at 40 mM K^+. B,C. Arithmetic means (± SEM, n = 40 - 52 cells) of the peak (B) and slope (C) of the change in intracellular Ca^{2+} concentrations following removal of external Na^+ (0 Na^+) and adding 2 mM Ca^{2+} at 40 mM K^+ in megakaryocytes without (white bars) and with (black bars) prior TGFß1 (60 ng/ml) treatment. *** (p<0.001) indicate statistically significant difference from untreated megakaryocytes (ANOVA).

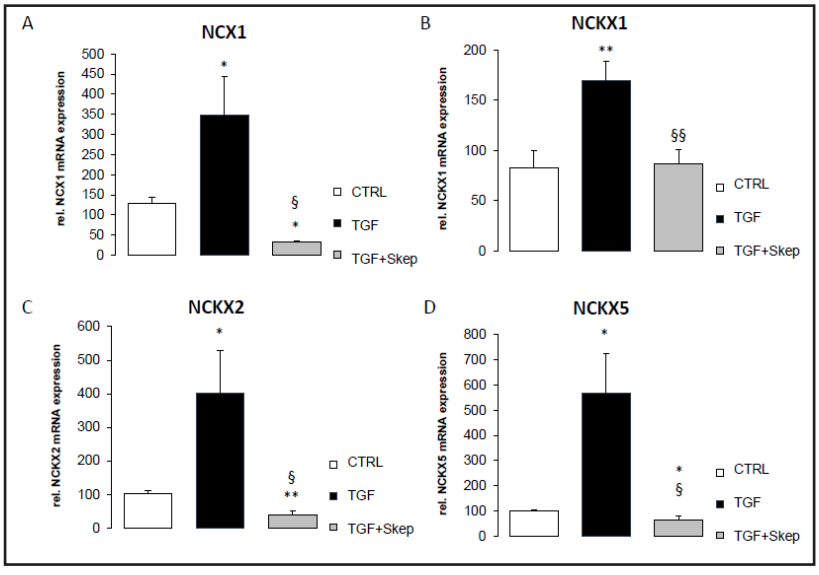

Fig. 6. Requirement of functional p38 kinase for TGFß1 sensitive expression of NCX1, NCKX1, NCKX2, and NCKX5 isoforms in megakaryocytes. A-D: Arithmetic means (± SEM, n = 4-11 preparations) of (A) NCX1, (B) NCKX1, (C) NCKX2, and (D) NCKX5 over GAPDH transcript levels in megakaryocytes without (white bars) and with prior TGFß1 treatment (60 ng/ml, 24 hours) in the absence (black bars) and presence (grey bars) of p38 kinase inhibitor Skepinone-L (1 µM). *(p<0.05), ** (p<0.01) indicates statistically significant difference from absence of TGFß1, $ (p<0.05), $$ (p<0.01) indicates statistically significant difference from absence of Skepinone-L (student´s t-test).

K^+ independent Na^+/Ca^{2+} exchanger activity by TGFß1 pretreatment was significantly blunted in the presence of p38 kinase inhibitor Skepinone-L. As shown in Fig. 8, the up-regulation of K^+ dependent Na^+/Ca^{2+} exchanger activity by TGFß1 pretreatment was again significantly blunted in the presence of p38 kinase inhibitor Skepinone-L.

The involvement of SGK1 in the up-regulation of Na^+/Ca^{2+} exchanger activity following TGFß1 treatment of megakaryocytes was tested by application of the SGK1 inhibitor GSK-650394 (10 µM). As illustrated in Fig. 9, the up-regulation of K^+ independent Na^+/Ca^{2+} exchanger activity by TGFß1 pretreatment was significantly blunted in the presence of SGK1 inhibitor GSK-650394. As shown in Fig. 10, the up-regulation of K^+ dependent Na^+/Ca^{2+}

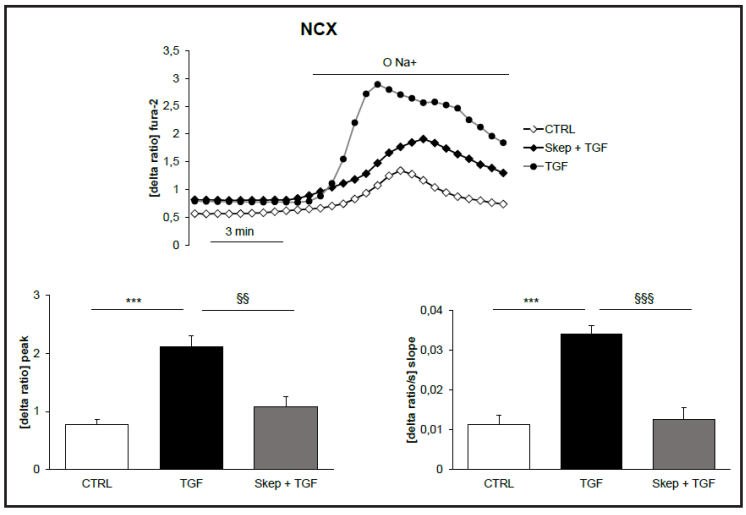

Fig. 7. Requirement of functional p38 kinase for TGFß1 sensitive NCX mediated Ca^{2+} entry in megakaryocytes. A. Representative original tracings showing intracellular Ca^{2+} concentrations in Fura-2/AM loaded megakaryocytes without TGFß1 treatment (open diamonds) and with prior 24 h treatment with 60 ng/ml TGFß1 alone (closed circles) or TGFß1 with p38 kinase inhibitor Skepinone-L (1 µM, closed diamonds) prior to and following removal of external Na^+ (0 Na^+) and adding 2 mM Ca^{2+} at 0 mM K^+. B,C. Arithmetic means (± SEM, n = 32 - 36 cells) of the peak (B) and slope (C) of the change in intracellular Ca^{2+} concentrations following removal of external Na^+ (0 Na^+) and adding 2 mM Ca^{2+} at 0 mM K^+ in megakaryocytes without (white bars) and with prior treatment with TGFß1 alone (black bars) or with TGFß1 and p38 kinase inhibitor Skepinone-L (grey bars). *** ($p<0.001$) indicate statistically significant difference from untreated megakaryocytes (ANOVA), §§ ($p<0.01$), §§§ ($p<0.001$) indicate statistically significant difference from megakaryocytes treated with TGFß1 alone.

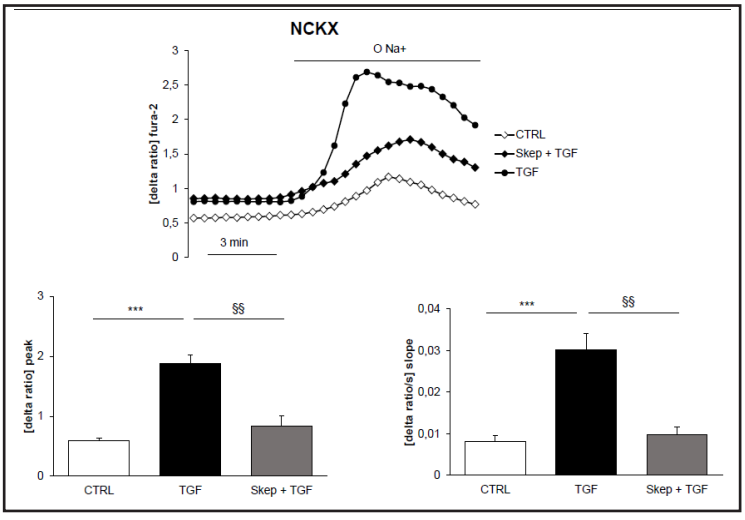

Fig. 8. Requirement of functional p38 kinase for TGFß1 sensitive NCKX mediated Ca^{2+} entry in megakaryocytes. A. Representative original tracings showing intracellular Ca^{2+} concentrations in Fura-2/AM loaded megakaryocytes without TGFß1 treatment (open diamonds) and with prior 24 h treatment with 60 ng/ml TGFß1 alone (closed circles) or TGFß1 with p38 kinase inhibitor Skepinone-L (1 µM, closed diamonds) prior to and following removal of external Na^+ (0 Na^+) and adding 2 mM Ca^{2+} at 40 mM K^+. B,C. Arithmetic means (± SEM, n = 32 - 36 cells) of the peak (B) and slope (C) of the change in intracellular Ca^{2+} concentrations following removal of external Na^+ (0 Na^+) and adding 2 mM Ca^{2+} at 40 mM K^+ in megakaryocytes without (white bars) and with prior treatment with TGFß1 alone (black bars) or with TGFß1 and p38 kinase inhibitor Skepinone-L (grey bars). *** ($p<0.001$) indicate statistically significant difference from untreated megakaryocytes (ANOVA), §§ ($p<0.01$) indicate statistically significant difference from megakaryocytes treated with TGFß1 alone.

exchanger activity by TGFß1 pretreatment was again significantly blunted in the presence of SGK1 inhibitor GSK-650394.

The involvement of NFκB in the up-regulation of Na^+/Ca^{2+} exchanger activity following TGFß1 treatment of megakaryocytes was tested by application of the NFκB inhibitor Wogonin (100 µM). As illustrated in Fig. 11, the up-regulation of K^+ independent Na^+/Ca^{2+} exchanger activity by TGFß1 pretreatment was significantly blunted in the presence of NFκB inhibitor Wogonin. As shown in Fig. 12, the up-regulation of K^+ dependent Na^+/Ca^{2+} exchanger activity by TGFß1 pretreatment was again significantly blunted in the presence of NFκB inhibitor Wogonin.

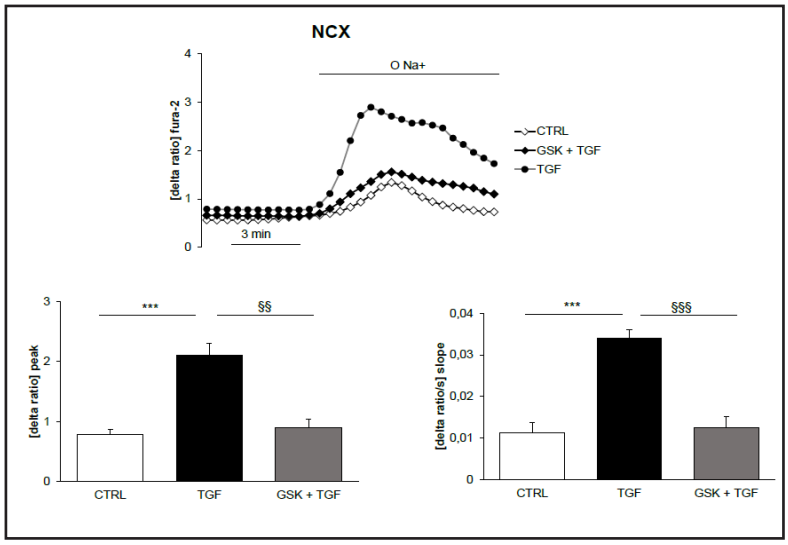

Fig. 9. Requirement of functional SGK1 for TGFß1 sensitive NCX mediated Ca^{2+} entry in megakaryocytes. A. Representative original tracings showing intracellular Ca^{2+} concentrations in Fura-2/AM loaded megakaryocytes without TGFß1 treatment (open diamonds) and with prior 24 h treatment with 60 ng/ml TGFß1 alone (closed circles) or TGFß1 with SGK1 inhibitor GSK-650394 (10 µM, closed diamonds) prior to and following removal of external Na^+ (0 Na^+) and adding 2 mM Ca^{2+} at 0 mM K^+. B,C. Arithmetic means (± SEM, n = 34 - 36 cells) of the peak (B) and slope (C) of the change in intracellular Ca^{2+} concentrations following removal of external Na^+ (0 Na^+) and adding 2 mM Ca^{2+} at 0 mM K^+ in megakaryocytes without (white bars) and with prior treatment with TGFß1 alone (black bars) or with TGFß1 and with SGK1 inhibitor GSK-650394 (10 µM) (grey bars). *** ($p<0.001$) indicate statistically significant difference from untreated megakaryocytes (ANOVA), §§§ ($p<0.001$), §§ ($p<0.01$) indicate statistically significant difference from megakaryocytes treated with TGFß1 alone.

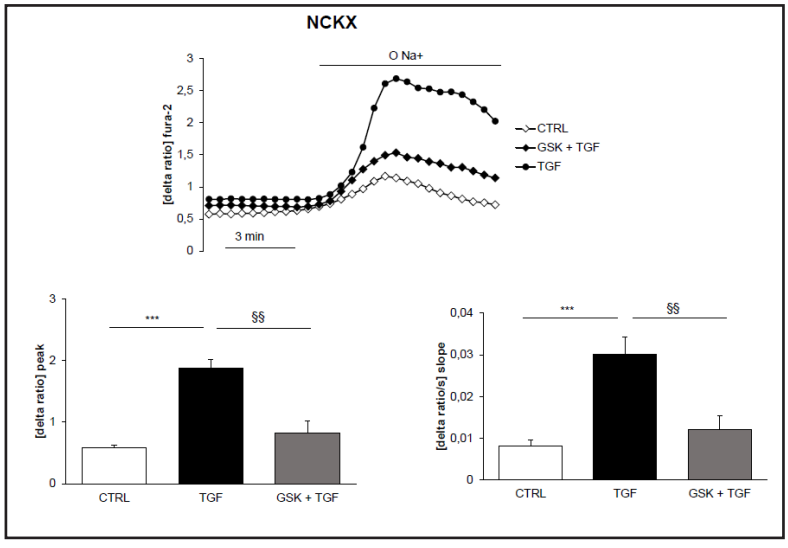

Fig. 10. Requirement of functional p38 kinase for TGFß1 sensitive NCKX mediated Ca^{2+} entry in megakaryocytes. A. Representative original tracings showing intracellular Ca^{2+} concentrations in Fura-2/AM loaded megakaryocytes without TGFß1 treatment (open diamonds) and with prior 24 h treatment with 60 ng/ml TGFß1 alone (closed circles) or TGFß1 with SGK1 inhibitor GSK-650394 (10 µM, closed diamonds) prior to and following removal of external Na^+ (0 Na^+) and adding 2 mM Ca^{2+} at 40 mM K^+. B,C. Arithmetic means (± SEM, n = 34 - 36 cells) of the peak (B) and slope (C) of the change in intracellular Ca^{2+} concentrations following removal of external Na^+ (0 Na^+) and adding 2 mM Ca^{2+} at 40 mM K^+ in megakaryocytes without (white bars) and with prior treatment with TGFß1 alone (black bars) or with TGFß1 and with SGK1 inhibitor GSK-650394 (grey bars). *** ($p<0.001$) indicate statistically significant difference from untreated megakaryocytes (ANOVA), §§ ($p<0.01$) indicate statistically significant difference from megakaryocytes treated with TGFß1 alone.

Discussion

The present study confirms the previous observation [21] that TGFß1 is a powerful stimulator of Na^+/Ca^{2+} exchange in megakaryocytes. The carriers may accomplish Ca^{2+} extrusion at high intracellular Ca^{2+} concentrations and/or hyperpolarized cell membrane potential,

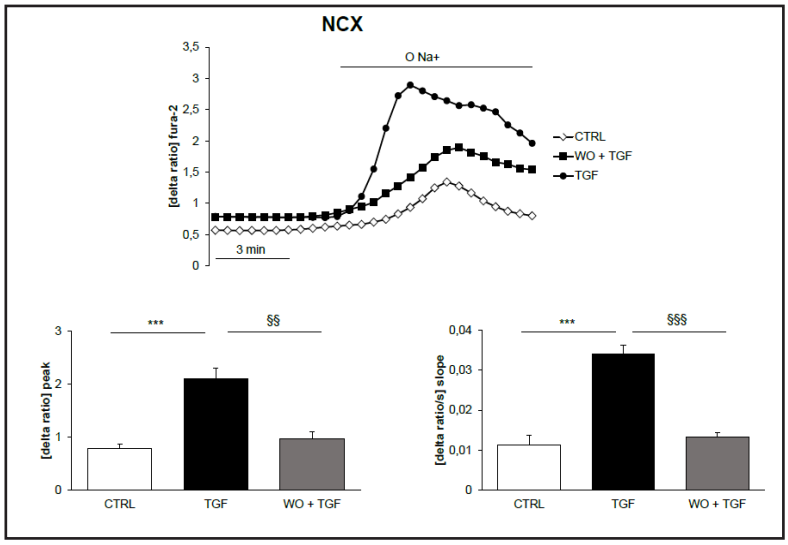

Fig. 11. Requirement of functional NF-κB for TGFß1 sensitive NCX mediated Ca^{2+} entry in megakaryocytes. A. Representative original tracings showing intracellular Ca^{2+} concentrations in Fura-2/AM loaded megakaryocytes without TGFß1 treatment (open diamonds) and with prior 24 h treatment with 60 ng/ml TGFß1 alone (closed circles) or TGFß1 with NF-κB inhibitor Wogonin (100 µM, closed squares) prior to and following removal of external Na^+ (0 Na^+) and adding 2 mM Ca^{2+} at 0 mM K^+. B,C. Arithmetic means (± SEM, n = 31 - 39 cells) of the peak (B) and slope (C) of the change in intracellular Ca^{2+} concentrations following removal of external Na^+ (0 Na^+) and adding 2 mM Ca^{2+} at 0 mM K^+ in megakaryocytes without (white bars) and with prior treatment with TGFß1 alone (black bars) or with TGFß1 and with NF-κB inhibitor Wogonin (100 µM) (grey bars). *** (p<0.001) indicate statistically significant difference from untreated megakaryocytes (ANOVA), §§ (p<0.01) §§§ (p<0.001) indicate statistically significant difference from megakaryocytes treated with TGFß1 alone.

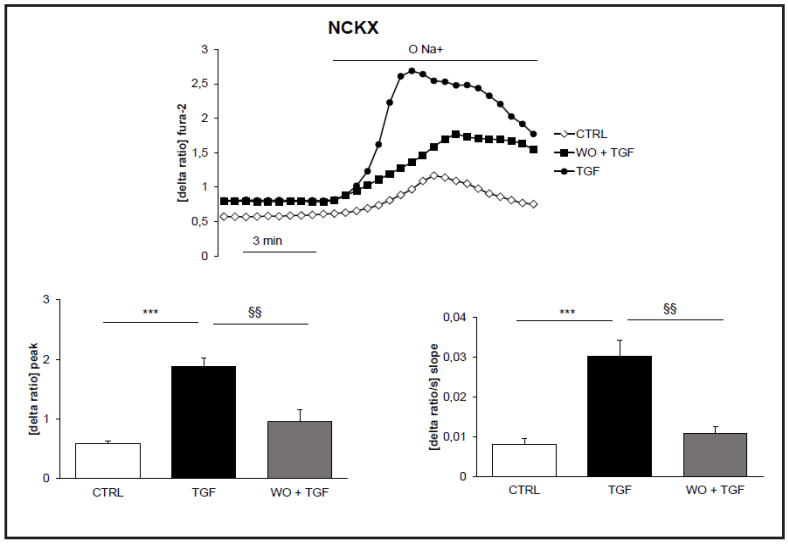

Fig. 12. Requirement of functional NF-κB for TGFß1 sensitive NCKX mediated Ca^{2+} entry in megakaryocytes. A. Representative original tracings showing intracellular Ca^{2+} concentrations in Fura-2/AM loaded megakaryocytes without TGFß1 treatment (open diamonds) and with prior 24 h treatment with 60 ng/ml TGFß1 alone (closed circles) or TGFß1 with NF-κB inhibitor Wogonin (100 µM, closed squares) prior to and following removal of external Na^+ (0 Na^+) and adding 2 mM Ca^{2+} at 40 mM K^+. B,C. Arithmetic means (± SEM, n = 31 - 39 cells) of the peak (B) and slope (C) of the change in intracellular Ca^{2+} concentrations following removal of external Na^+ (0 Na^+) and adding 2 mM Ca^{2+} at 40 mM K^+ in megakaryocytes without (white bars) and with prior treatment with TGFß1 alone (black bars) or with TGFß1 and with NF-κB inhibitor Wogonin (grey bars). *** (p<0.001) indicate statistically significant difference from untreated megakaryocytes (ANOVA), §§ (p<0.01) indicate statistically significant difference from megakaryocytes treated with TGFß1 alone.

but may mediate Ca^{2+} entry during high intracellular Na^+ concentration and/or depolarized cell membrane [22, 36-38]. TGFß1 up-regulates both, Ca^{2+} entry via Orai1 [20] and Ca^{2+} entry or Ca^{2+} extrusion by Na^+/Ca^{2+} exchange [21].

Simultaneous stimulation of Ca^{2+} entry via Orai1 and Ca^{2+} extrusion by Na^+/Ca^{2+} exchangers may trigger Ca^{2+} oscillations [39] which contribute to the regulation of diverse cellular functions [25, 40-43]. Ca^{2+} oscillations are required for the entrance into the S and the

M phase of the cell cycle [44, 45] and confer cell survival [46, 47]. Ca^{2+} oscillations critically depend on timely entry and timely extrusion of Ca^{2+}. In the absence of Ca^{2+} extrusion Ca^{2+} entry leads to sustained increase of cytosolic Ca^{2+} activity with stimulation of apoptosis [40, 42, 48-56].

The present observations define the NCX and NCKX isoforms involved in the TGFß1-induced up-regulation of Na^+/Ca^{2+} exchange and shed light on the signalling involved. Similar to what has been observed in the regulation of Orai1 and SOCE [20], the effect of TGFß1 is blunted or even abrogated in the presence of p38 kinase inhibitor Skepinone-L, of SGK1 inhibitor GSK-650394 and of NFκB inhibitor Wogonin. TGFß1 is thus presumably effective by upregulating p38 kinase [18] with subsequent up-regulation of SGK1 [15], which in turn activates nuclear factor NFκB [15, 16].

In addition to directly modify Ca^{2+} transport, TGFß1 may indirectly influence Na^+/Ca^{2+} exchange by up-regulating the Na^+/K^+ ATPase in megakaryocytes thus enhancing the Na^+ gradient and potential difference driving Ca^{2+} extrusion via the Na^+/Ca^{2+} exchange [57]. The effect of TGFß1 on Na^+/K^+ ATPase similarly involves p38 kinase, SGK1 and NF-κB [57].

TGFß1 is produced by megakaryocytes [58, 59] and required for megakaryocyte maturation and platelet formation [19]. The growth factor stimulates expression of bone marrow stromal thrombopoietin [19], which in turn stimulates the expression of megakaryocytic TGF-beta receptors [19]. TGFß1 is thus a powerful regulator of megakaryopoiesis [19] and excessive TGFß1 expression leads to myelofibrosis [59].

Besides its putative impact on megakaryocyte proliferation, maturation and survival, activation of megakaryocytes with TGFß1 yields platelets with enhanced Orai1 dependent Ca^{2+} entry as well as NCX and NCKX dependent Ca^{2+} entry and extrusion, which presumably impacts on the platelet response to activators such as thrombin or collagen related peptide [15].

In conclusion, TGFß1 up-regulates the Na^+/Ca^{2+} exchanger isoforms NCX1, NCKX1, NCKX2 and NCKX5, and thus does not only up-regulate store operated Ca^{2+} entry but as well Ca^{2+} extrusion by the Na^+/Ca^{2+} exchanger isoforms. The signalling for the regulation of both, SOCE and Na^+/Ca^{2+} exchange involves p38 kinase, SGK1 and NFκB.

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Disclosure Statement

None.

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