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Up-regulation of megakaryocytic Na^+/Ca^{2+} exchange in klotho-deficient mice

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ABSTRACT

The active form of vitamin D, $1,25(OH)_2D_3$, is a powerful regulator of cytosolic Ca^{2+}-concentration ($[Ca^{2+}]_i$) in a variety of cell types. The formation of $1,25(OH)_2D_3$ is inhibited by FGF23, an effect requiring presence of klotho. $1,25(OH)_2D_3$ plasma levels are excessive in klotho-deficient mice (*kl/kl*). A previous study revealed that klotho-deficiency is followed by decreased activation of platelets, an effect at least in part due to blunted store operated Ca^{2+} entry (SOCE). In other cell types $1,25(OH)_2D_3$ has been shown to up-regulate the Na^+/Ca^{2+}-exchanger, which could, depending on cell membrane potential and cytosolic Na^+ concentration, either decrease or increase $[Ca^{2+}]_i$. The present study explored whether Na^+/Ca^{2+}-exchanger activity is different in megakaryocytes isolated from *kl/kl* mice than in megakaryocytes isolated from wild type mice. Na^+/Ca^{2+}-exchanger induced currents were determined by whole cell patch clamp and the Na^+/Ca^{2+}-exchanger induced alterations of $[Ca^{2+}]_i$ by Fura-2 fluorescence. As a result, the inward current and the increase of $[Ca^{2+}]_i$ following replacement of extracellular Na^+ by NMDG were higher in *kl/kl* megakaryocytes than in wild type megakaryocytes, a difference abrogated by treatment of the mice with low Vitamin D diet. Pretreatment of wild type megakaryocytes with $1,25(OH)_2D_3$ (100 nM, 48 h) was followed by enhancement of both, inward current and increase of $[Ca^{2+}]_i$ following replacement of extracellular Na^+ by NMDG. In conclusion, the present observations reveal a powerful stimulating effect of $1,25(OH)_2D_3$ on Na^+/Ca^{2+}-exchanger activity in megakaryocytes.

1. Introduction

Platelet activation is essential for primary hemostasis, but by the same token contributes to vascular inflammation and arterial thrombotic occlusion [1–4]. Platelets are activated by an increase of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) [5]. Mechanisms increasing platelet $[Ca^{2+}]_i$ include inositol-1,4,5-triphosphate (IP_3)-mediated release of Ca^{2+} from intracellular stores with subsequent stimulation of store operated calcium entry (SOCE) [6–8]. SOCE is down-regulated by $1,25(OH)_2$ vitamin D_3 ($1,25(OH)_2D_3$), the biologically active metabolite of vitamin D [9]. Megakaryocytes and blood platelets do express the vitamin D receptor and are thus expected to be sensitive to its ligand $1,25(OH)_2D_3$ [10]. $1,25(OH)_2D_3$ formation is under tight control of klotho [11]. Klotho deficiency in mice accelerates the appearance of age-related disorders and dramatically decreases the life span [12–14], effects largely due to excessive $1,25(OH)_2D_3$ formation and thus reversed by vitamin D-deficient diet [11,15].

Klotho deficiency has a strong impact on Ca^{2+}-dependent platelet activation and thrombus formation, effects fully reversed by normalization of $1,25(OH)_2D_3$ levels with vitamin D deficient diet [9]. The klotho gene is expressed predominantly in the kidney and the choroid plexus, but not in hematopoietic organs including bone marrow, thymus and spleen [12,16]. Moreover, klotho is not expressed in platelets and klotho deficiency is thus effective by excessive formation of $1,25(OH)_2D_3$. In other cell types, however, $1,25(OH)_2D_3$ has been found to counteract an increase of $[Ca^{2+}]_i$ by increasing Na^+/Ca^{2+} exchanger activity [17].

The present study explored whether Na^+/Ca^{2+} exchanger activity in megakaryocytes is modified by klotho deficiency. To this

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end megakaryocytes were isolated from klotho-deficient mice and wild type mice. Na^+/Ca^{2+} exchanger activity was quantified by both, whole cell patch clamp recordings and Fura-2-fluorescence.

2. Materials and methods

2.1. Mice

Megakaryocytes were isolated from klotho-deficient mice (*klotho*^{hm}) and their wild-type littermates (*klotho*$^{+/+}$). Origin of the mice, breeding, and genotyping were described previously [12]. Congenic strains of Klotho-deficient mice were produced by repeated backcrosses (>9 generations) to the 129 inbred strains and used in this study. All animal experiments were performed according to the German animal protection law and approved by the local authorities. Male and female mice were studied at the age of 6–8 wk. The mice had access to water ad libitum and to control food (Altromin 1310) or vitamin D-deficient diet (Altromin C1017).

2.2. Isolation and culture of murine megakaryocytes

For the isolation of megakaryocytes, bone marrow cells were harvested by flushing the femurs and tibiae with phosphate-buffered saline (Gibco). The obtained cells were separated over Percoll (GE Healthcare) and cultured in specific growth medium (MethoCult®, Stemcell) containing 10% fetal bovine serum (Gibco), 20% IMDM with Glutamax (Gibco) and 50 ng/ml thrombopoietin (Invitrogen). After 7 days differentiation into megakaryocytes was tested by microscopy as well as glyocoprotein Ib (GPIbα) staining and western blotting.

2.3. Measurement of intracellular Ca^{2+}

Fluorescence measurements were carried out with an inverted phase-contrast microscope (Axiovert 100, Zeiss, Oberkochen, Germany) [18]. Cells were excited alternatively at 340 or 380 nm and the light was deflected by a dichroic mirror into either the objective (Fluar 40 × /1.30 oil, Zeiss, Oberkochen, Germany) or a camera (Proxitronic, Bensheim, Germany). Emitted fluorescence intensity was recorded at 505 nm and data acquisition was accomplished by using specialized computer software (Metafluor, Universal Imaging, Downingtown, USA). As a measure for the increase of cytosolic Ca^{2+} activity, the slope and peak of the changes in the intracellular Ca^{2+} ratio were calculated for each experiment.

The cells were loaded with Fura-2/AM (2 μM, Molecular Probes, Goettingen, Germany) for 30 min at 37 °C. Changes in cytosolic Ca^{2+}

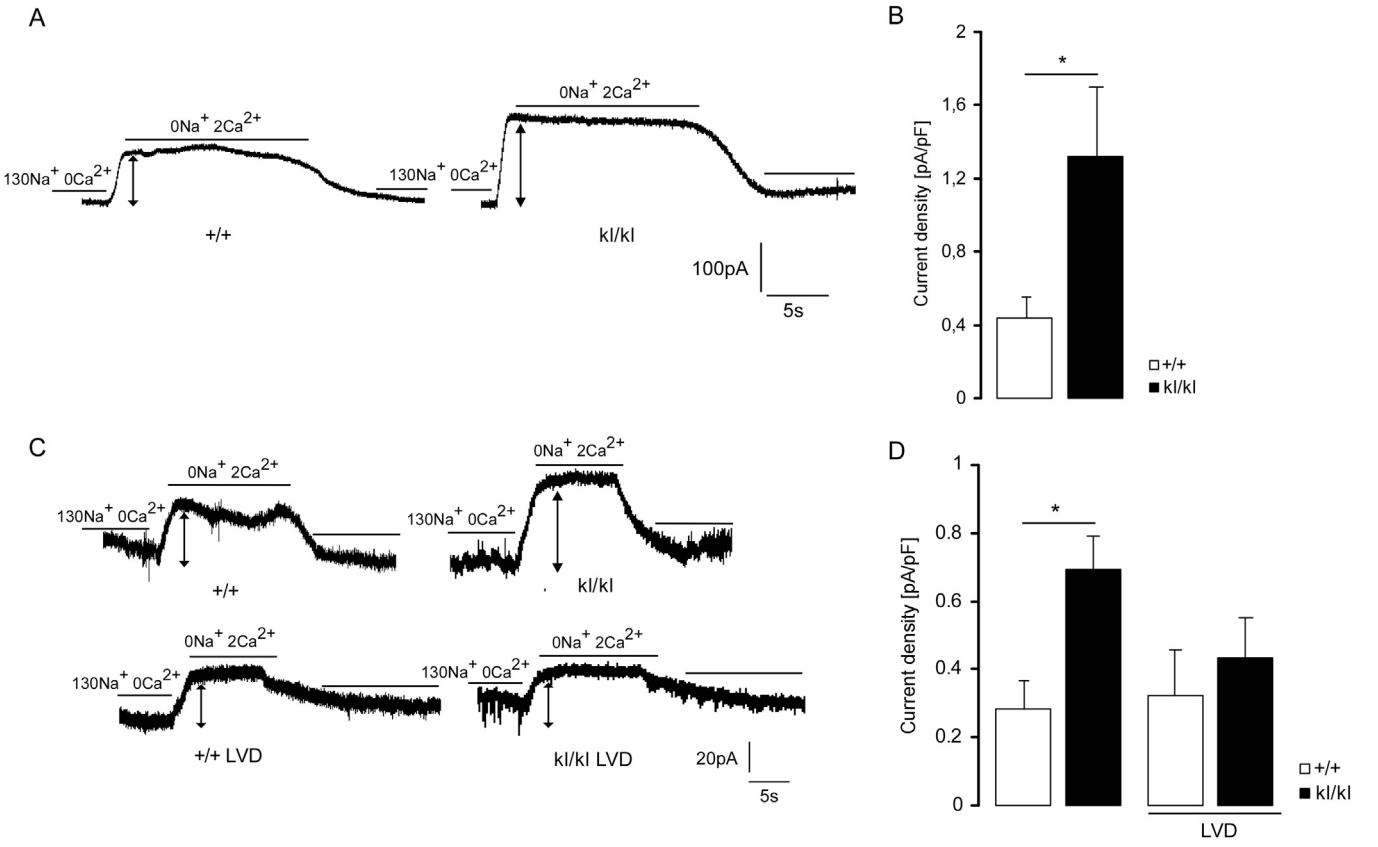

Fig. 1. Na^+/Ca^{2+} exchanger currents in megakaryocytes from *kl/kl* and *wt* mice with control and low vitamin D diet. A. Whole cell currents in megakaryocytes from klotho hypomorphic (*kl/kl*) mice (right) and wild type (*wt*) mice (left) recorded at −80 mV during the switch between external solutions from 130 mM Na^+ and no Ca^{2+} (130 Na^+ 0 Ca^{2+}) to 2 mM Ca^{2+} and no Na^+ (0 Na^+ 2 Ca^{2+}). The internal solution contained 1 μM free Ca^{2+}, 120 mM Na^+, and 40 mM K^+. Cesium and TEA^+ were present in the solutions to block K^+ channel currents. B. Mean (±SEM, n = 10/8 cells) current density changes (ΔI, pA/pF) in megakaryocytes from *kl/kl* mice (black bar) and *wt* mice (white bar) at −80 mV induced by the switch between external solutions from 130 Na^+, 0 Ca^{2+} to 0 Na^+, 2 Ca^{2+}. The solutions were as in A. *($p < 0.05$) indicates significant difference from *wt* megakaryocytes (two-tailed unpaired *t*-test). C. Whole cell currents in megakaryocytes from wild type (*wt*) mice (left) and klotho hypomorphic (*kl/kl*) mice (right) without (upper panels) and with (lower panels) low vitamin D diet recorded at −80 mV during the switch between external solutions from 130 mM Na^+ and no Ca^{2+} (130 Na^+ 0 Ca^{2+}) to 2 mM Ca^{2+} and no Na^+ (0 Na^+ 2 Ca^{2+}). The internal solution contained 1 μM free Ca^{2+}, 120 mM Na^+, and 40 mM K^+. Cesium and TEA^+ were present in the solutions to block K^+ channel currents. D. Mean (±SEM, n = 11/32/14/9 cells) current density changes (ΔI, pA/pF) in megakaryocytes from *kl/kl* mice (black bars) and *wt* mice (white bars) without (left bars) and with (right bars) low vitamin D diet at −80 mV induced by the switch between external solutions from 130 Na^+, 0 Ca^{2+} to 0 Na^+, 2 Ca^{2+}. The solutions were as in C. *($p < 0.05$) indicates significant difference from *wt* megakaryocytes (ANOVA).

activity were monitored to study Na^+/Ca^{2+} exchange mechanisms upon removal of extracellular Na^+. N-methyl-D-glucamine (NMDG) was used to replace Na^+. To measure Na^+/Ca^{2+} exchange the standard solution contained (in mM): 90 NaCl, 0 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, 5 glucose, pH 7.4 and the Na^+-free solution contained (in mM): 90 NMDG, 0 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, 10 glucose, pH 7.4.

2.4. Patch clamp

Patch clamp experiments were performed at room temperature in voltage-clamp, fast-whole-cell mode as described previously [19]. The cells were continuously superfused through a flow system inserted into the dish [20]. The bath was grounded via a bridge filled with NaCl Ringer solution. Borosilicate glass pipettes (1–3 MOhm tip resistance; GC 150 TF-10, Clark Medical Instruments, Pangbourne, UK) manufactured by a microprocessor-driven DMZ puller (Zeitz, Augsburg, Germany) were used in combination with an MS314 electrical micromanipulator (MW, Märzhäuser, Wetzlar, Germany). The currents were recorded by an EPC-9 amplifier (Heka, Lambrecht, Germany) using Pulse software (Heka) and an ITC-16 Interface (Instrutech, Port Washington, N.Y., USA). Voltage clamp steps (2 min) were applied to the potential of −80 mV from the holding potential of 0 mV with an acquisition frequency of 10 and 3 kHz low-pass filtered [21]. The liquid junction potential ΔE between the pipette and the bath solutions and between the salt bridge and the bath solutions was estimated as described previously [22]. Data were corrected for the estimated ΔE values.

To measure Na^+/Ca^{2+} exchanger-mediated currents, an Na^+-based pipette solution was used composed of 120 mM NaCl, 40 mM KCl, 20 mM TEA-Cl, 2 mM $MgCl_2$, 2 mM Mg-ATP, 10 mM HEPES (pH 7.2, CsOH), and 1 μM free Ca^{2+}. The external solution contained 130 mM NaCl, 20 mM TEA-Cl, 2 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES (pH 7.2, CsOH), 0.5 mM EGTA, and 40 mM KCl. Na^+/Ca^{2+} exchange currents were elicited by switching to a bath solution in which Na^+ was substituted by $NMDG^+$.

2.5. Statistics

Data are provided as means ± SEM, *n* represents the number of independent experiments. All data were tested for significance using Student's unpaired two-tailed *t*-test or ANOVA and only results with $p < 0.05$ were considered statistically significant.

3. Results

The present study explored whether klotho deficiency influences the Na^+/Ca^{2+} exchanger activity in megakaryocytes. In a first approach whole cell currents were recorded in megakaryocytes utilizing patch clamp in order to elucidate the currents generated by Na^+/Ca^{2+} exchanger activity of megakaryocytes. As illustrated in Fig. 1A, B, Na^+/Ca^{2+} exchanger currents were observed in megakaryocytes isolated from *kl/kl* mice and *wt* mice. The currents were significantly higher in megakaryocytes isolated from *kl/kl* mice than in megakaryocytes isolated from *wt* mice (Fig. 1A, B).

A next series of experiments explored whether the differences of Na^+/Ca^{2+} exchanger currents between megakaryocytes isolated from *kl/kl* mice and megakaryocytes isolated from *wt* mice were the result of the excessive $1,25(OH)_2D_3$ concentrations in *kl/kl* mice. To this end, *kl/kl* mice and *wt* mice were treated with a low vitamin D diet known to dissipate the differences of $1,25(OH)_2D_3$ plasma concentrations between *kl/kl* mice and *wt* mice [9]. As shown in Fig. 1C, D, the differences of Na^+/Ca^{2+} exchanger currents between megakaryocytes isolated from *kl/kl* mice and megakaryocytes isolated from *wt* mice were virtually abolished by prior treatment of the mice with low vitamin D diet. Thus, klotho deficiency upregulated Na^+/Ca^{2+} exchanger currents most likely by increasing plasma $1,25(OH)_2D_3$ concentration.

To more directly test for effects of $1,25(OH)_2D_3$ on Na^+/Ca^{2+} exchanger currents additional experiments explored whether pretreatment of wild type megakaryocytes with $1,25(OH)_2D_3$ (100 nM, 48 h) mimics the effect of klotho deficiency. As illustrated in Fig. 2, the Na^+/Ca^{2+} exchanger currents were significantly higher in $1,25(OH)_2D_3$ treated wild type megakaryocytes than in untreated wild type megakaryocytes.

Fura-2 fluorescence has been employed for the determination of intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) to further elucidate the impact of klotho deficiency on Na^+/Ca^{2+} exchanger activity of megakaryocytes. To this end, megakaryocytes were loaded with Fura-2/AM prior to the experiments. The increase of $[Ca^{2+}]_i$ following removal of external Na^+ was taken as evidence for Na^+/Ca^{2+} exchanger activity. As illustrated in Fig. 3A, B, the increases of slope and peak Fura-2 fluorescence ratio values were both significantly higher in megakaryocytes isolated from *kl/kl* mice than in megakaryocytes isolated from *wt* mice.

As illustrated in Fig. 3C, D, the differences of Ca^{2+} entry following extracellular Na^+ removal between megakaryocytes isolated from *kl/kl* mice and megakaryocytes isolated from *wt* mice were virtually abolished by treatment with low vitamin D diet. Thus, klotho deficiency was most likely effective by increasing plasma $1,25(OH)_2D_3$ concentration.

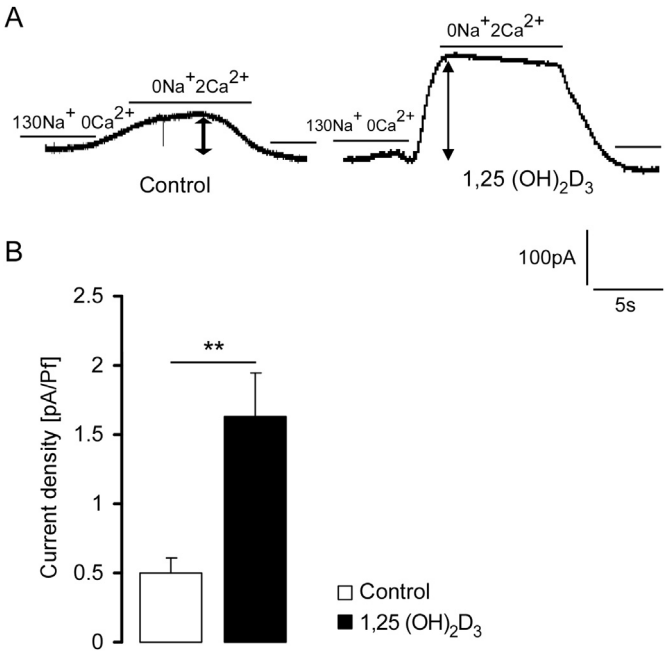

Fig. 2. Na^+/Ca^{2+} exchanger currents in megakaryocytes from *wt* mice without and with $1,25(OH)_2D_3$ treatment. A. Whole cell currents in megakaryocytes from wild type (*wt*) mice without (left) and with (right) prior (48 h) treatment with $1,25(OH)_2D_3$ (100 nM) recorded at −80 mV during the switch between external solutions from 130 mM Na^+ and no Ca^{2+} (130 Na^+ 0 Ca^{2+}) to 2 mM Ca^{2+} and no Na^+ (0 Na^+ 2 Ca^{2+}). The internal solution contained 1 μM free Ca^{2+}, 120 mM Na^+, and 40 mM K^+. Cesium and TEA^+ were present in the solutions to block K^+ channel currents. B. Mean (±SEM, n = 12/5 cells) current density changes (ΔI, pA/pF) in megakaryocytes from *wt* mice without (white bar) and with (black bar) prior (48 h) treatment with $1,25(OH)_2D_3$ (100 nM) recorded at −80 mV induced by the switch between external solutions from 130 Na^+, 0 Ca^{2+} to 0 Na^+, 2 Ca^{2+}. The solutions were as in A. **($p < 0.01$) indicates significant difference from *wt* megakaryocytes without $1,25(OH)_2D_3$ treatment (two-tailed unpaired *t*-test).

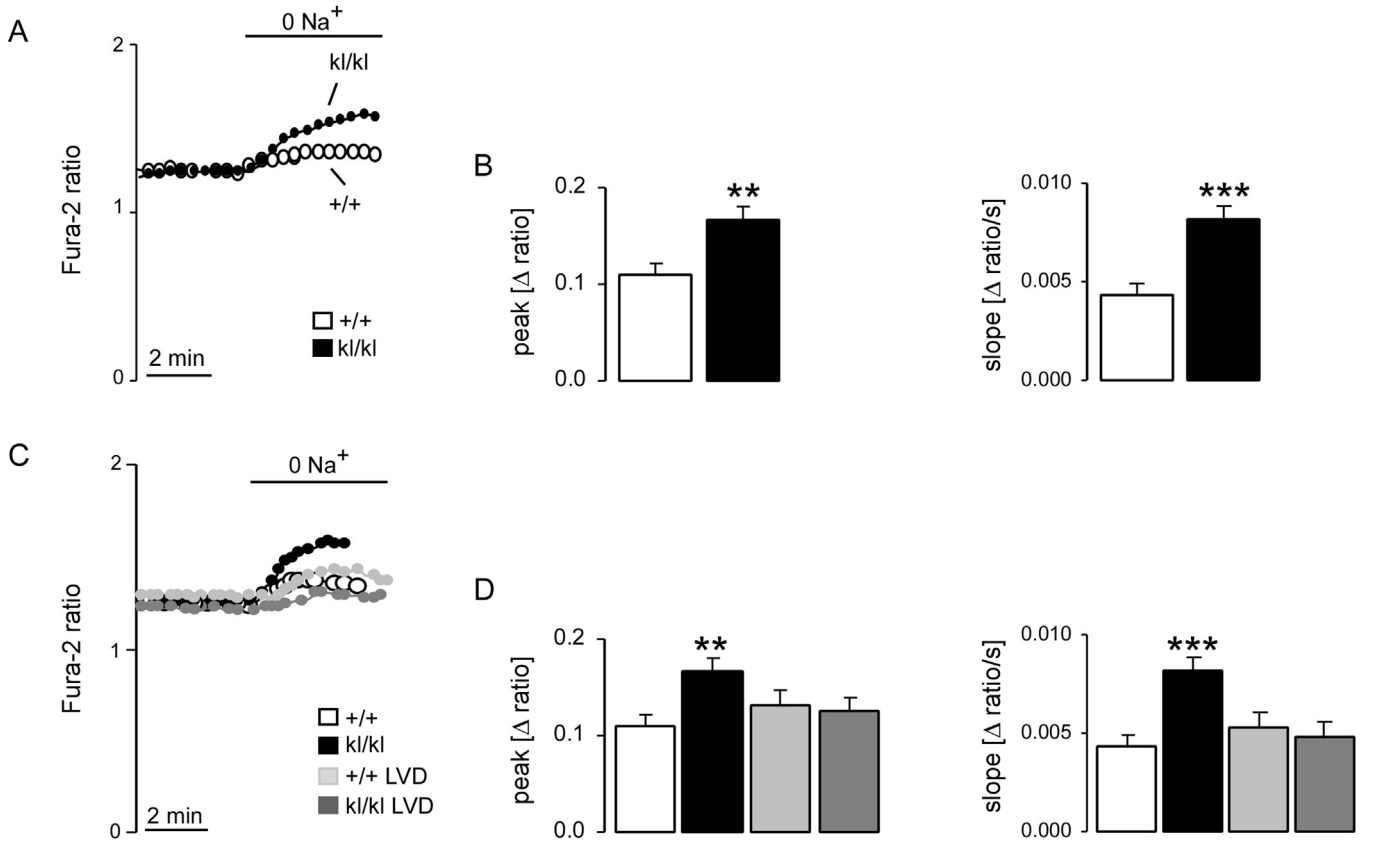

Fig. 3. Na^+/Ca^{2+} exchanger induced Ca^{2+} entry into megakaryocytes from *kl/kl* and *wt* mice with control and low vitamin D diet. A. Representative original tracings showing Fura-2 fluorescence ratio reflecting intracellular Ca^{2+} concentrations in Fura-2/AM loaded megakaryocytes from klotho hypomorphic (*kl/kl*) mice (black circles) and wild type (*wt*) mice (white circles) prior to and following removal of external Na^+ (0 Na^+). B. Mean (±SEM, n = 31/32 cells) of the peak value (left) and slope (right) of the change in Fura-2 fluorescence ratio in megakaryocytes from klotho hypomorphic (*kl/kl*) mice (black bars) and wild type (*wt*) mice (white bars) following removal of external Na^+. **($p < 0.01$), ***($p < 0.001$) indicates significant difference from *wt* megakaryocytes (two-tailed unpaired *t*-test). C. Representative original tracings showing Fura-2 fluorescence ratio reflecting intracellular Ca^{2+} concentrations in Fura-2/AM loaded megakaryocytes from klotho hypomorphic (*kl/kl*) mice and wild type (*wt*) mice under control diet and under low vitamin D diet prior to and following removal of external Na^+ (0 Na^+). D. Mean (±SEM, n = 31/22/32/16 cells) of the peak value (left) and slope (right) of the change in Fura-2 fluorescence ratio following removal of external Na^+ in megakaryocytes from klotho hypomorphic (*kl/kl*) mice under control diet (black bars) or low vitamin D diet (dark gray bars) and from wild type (*wt*) mice under control diet (white bars) or low vitamin D diet (light gray bars). **($p < 0.01$), ***($p < 0.001$) indicates significant difference from respective *wt* megakaryocytes (ANOVA).

Additional experiments thus explored whether pretreatment of wild type megakaryocytes with $1,25(OH)_2D_3$ (100 nM, 48 h) mimics the effect of klotho deficiency on Ca^{2+} entry into megakaryocytes following extracellular Na^+ removal. As illustrated in Fig. 4, the increase of $[Ca^{2+}]_i$ following removal of external Na^+ was significantly higher in $1,25(OH)_2D_3$ treated wild type megakaryocytes than in untreated wild type megakaryocytes.

4. Discussion

The present observations reveal a novel effect of klotho deficiency and $1,25(OH)_2D_3$ on megakaryocytes. The hormone $1,25(OH)_2D_3$ up-regulates Na^+/Ca^{2+} exchanger activity. The carrier has a dual role on intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$). At high intracellular Ca^{2+} concentration and/or hyperpolarized cell membrane potential the carrier mediates the extrusion of Ca^{2+} thus blunting the increase of $[Ca^{2+}]_i$, whereas at high intracellular Na^+ concentration and/or depolarized cell membrane potential the carrier operates in the reversed mode thus loading the cell with Ca^{2+} [17,23,24].

$1,25(OH)_2D_3$ has previously been shown to blunt the increase of $[Ca^{2+}]_i$ following activation of platelets [9]. The effect was at least partially due to downregulation of SOCE in megakaryocytes and blood platelets [9]. According to the present observations, accelerated Ca^{2+} extrusion by the up-regulated Na^+/Ca^{2+} exchanger presumably contributes to the blunted increase of $[Ca^{2+}]_i$ in activated platelets following $1,25(OH)_2D_3$ treatment.

Klotho is not expressed in platelets and klotho deficiency is effective by excessive formation of $1,25(OH)_2D_3$ [9]. Accordingly, restricted vitamin D intake fully reversed the platelet and megakaryocyte phenotype of klotho deficient mice, as it normalized the $1,25(OH)_2D_3$ levels [9]. Low vitamin D diet dissipates further phenotypical features of *kl/kl* mice and almost all abnormalities observed in those mice are reversed by dietary vitamin D restriction [11]. Besides inhibition of SOCE [9], $1,25(OH)_2D_3$ counteracts coagulation [25] by down-regulating tissue factor expression in monocytes [26]. Along those lines disruption of the VDR gene enhances thrombogenicity in mice [27].

Earlier observations have revealed the participation of $1,25(OH)_2D_3$ in the regulation, differentiation, biosynthetic activity, and function of hematopoietic cells [28,29]. $1,25(OH)_2D_3$ counteracts inflammation by suppressing cytokine and chemokine gene expression in endothelial cells, macrophages, dendritic cells and lymphocytes [28,30–32]. Vitamin D deficiency may lead to cardiovascular disease including ischemic heart disease and mortality from cardiovascular events [31,33–37]. Conversely, high levels of biologically active vitamin D apparently protect against ischemic heart disease and mortality from cardiovascular events [36,38,39]. Whether or not dietary vitamin D prevents ischemic cardiovascular events remains to be shown [40].

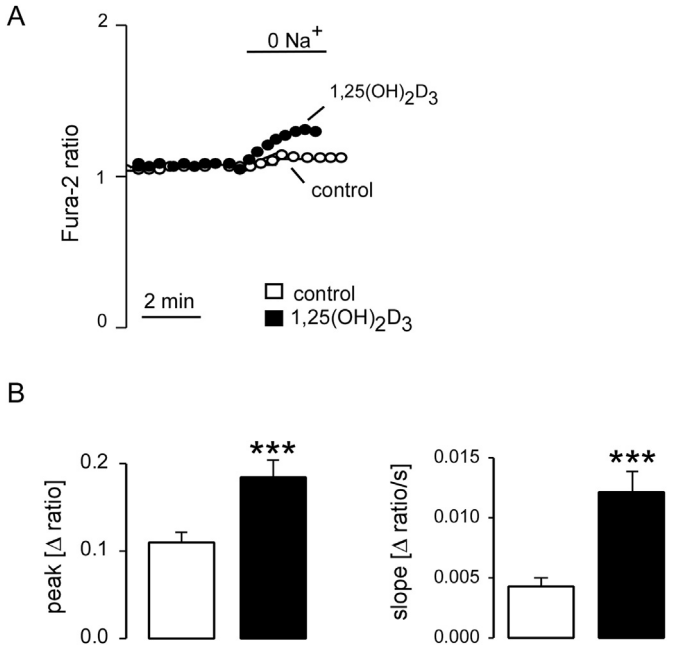

Fig. 4. Na^+/Ca^{2+} exchanger induced Ca^{2+} entry into megakaryocytes from *wt* mice without and with $1,25(OH)_2D_3$ treatment. A. Representative original tracings showing Fura-2 fluorescence ratio reflecting intracellular Ca^{2+} concentrations prior to and following removal of external Na^+ (0 Na^+) in Fura-2/AM loaded megakaryocytes from wild type (*wt*) mice without (white circles, control) and with (black circles) pretreatment of $1,25(OH)_2D_3$ (100 nM, 48 h). B. Mean (±SEM, n = 32/17 cells) of the peak value (left) and slope (right) of the change in Fura-2 fluorescence ratio following removal of external Na^+ in megakaryocytes from wild type (*wt*) mice without (white bars, control) and with (black bars) pretreatment of $1,25(OH)_2D_3$ (100 nM, 48 h). ***($p < 0.001$) indicates significant difference from untreated *wt* megakaryocytes (two-tailed unpaired *t*-test).

In summary, klotho deficiency and $1,25(OH)_2D_3$ up-regulate the Na^+/Ca^{2+} exchanger activity in megakaryocytes and thus influence megakaryocytic Ca^{2+} signaling not only through down-regulation of store operated Ca^{2+} entry but as well by augmenting the impact of the Na^+ electrochemical gradients on Ca^{2+} entry or exit.

Disclosure of conflict of interests

The authors state that they have no conflict of interest.

Acknowledgments

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Transparency document

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