Total β-adrenoceptor deficiency results in cardiac hypotrophy and negative inotropy

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SUMMARY

The present study investigated cardiac function in hearts of mice with total deficiency of the β_1-, β_2- and β_3-adrenoceptors (TKO) in comparison to wildtype mice (WT). We investigated cardiac morphology and echocardiographic function, measured protein expression of Ca^{2+}-regulatory proteins, SERCA 2a activity, myofibrillar function, and performed running wheel tests. Heart weight and heart-to-body weight ratio were significantly smaller in TKO as compared to WT. This was accompanied by a decrease in the size of the cardiomyocytes in TKO. Heart rate and ejection fraction were significantly diminished in TKO as compared to WT. Protein expression of SERCA 2a, the ryanodine receptor and the Na^+/Ca^{2+}-exchanger were similar in TKO and WT mice, but phospholamban protein expression was increased. PKA-dependent phosphorylation of phospholamban at serine 16 was absent and CaMKII-dependent phosphorylation at threonine 17 was decreased in TKO. All alterations were paralleled by a decrease in SERCA 2a-activity. A similar maximal calcium-dependent tension but an increased myofibrillar calcium-sensitivity was measured in TKO as compared to WT. We did not observe relevant functional impairments of TKO in running wheel tests. In the absence of β-agonistic stimulation, SERCA 2a activity is mainly regulated by alterations of phospholamban expression and phosphorylation. The decreased SERCA 2a activity following β-adrenoceptor deficiency may be partly compensated by an increased myofibrillar calcium-sensitivity.

KEYWORDS

beta-1 adrenoceptor, beta-2 adrenoceptor, beta-3 adrenoceptor, knockout, mice

INTRODUCTION

β-adrenergic stimulation is one of the main neurohumoral mechanisms modulating cardiac function in physiological (e.g. exercise) as well as pathophysiological conditions (e.g. heart failure). Three β-adrenoceptor (β-AR) subtypes have been described with the β_1- and β_2-adrenoceptors mediating positive inotropic reactions mainly by stimulating the G_s-protein/adenylate cyclase/protein kinase A pathway [for review see (Wallukat 2002)], whereas β_3-adrenergic stimulation is coupled to a cardiac release of nitric oxide via activation of the endothelial NO-synthase (Brixius *et al.* 2004, Gauthier *et al.* 1998, Gauthier *et al.* 1996, Moniotte *et al.* 2001), the functional implication of which has still to be clarified.

β-adrenergic positive inotropic effects are paralleled by an increase of intracellular systolic Ca^{2+}-concentrations due to an increase in the Ca^{2+}-influx through the L-type Ca^{2+}-channels (Bers 2002), which is subsequently followed by an increase in the Ca^{2+}-release out of the sarcoplasmic reticulum, as well as a decrease in the myofibrillar Ca^{2+}-sensitivity (Brixius *et al.* 2002, van der Velden *et al.* 2003) and an increase in the Ca^{2+}-accumulation by the sarcoplasmic reticulum (Bers 2002). The latter can be attributed to an increase in the activity of the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA 2a) due to an increased phosphorylation of serine 16 (PLB-Ser16) and threonine 17 (PLB-Thr17) phospholamban (PLB) (Kranias and Solaro 1982, MacLennan and Kranias 2003).

Only recently, a transgenic mouse model was described with chronic deficiency of all three β-adrenoceptor subtypes (Bachman *et al.* 2002, Bachman *et al.* 2004, Jimenez *et al.* 2002, Lee *et al.* 2008). The mice are viable and are characterized by cold

intolerance due to impaired adaptive responses of the brown adipose tissue to cold exposure. We were now interested in the regulation of the intracellular Ca^{2+}-homeostasis and myofibrillar function under conditions of chronic β-adrenergic deficiency. Therefore, we investigated cardiac morphology and echocardiographic function in total β-adrenoceptor knockout mice in comparison to their isogenic littermates. In addition, we measured protein expression of Ca^{2+}-regulatory proteins, SERCA 2a activity, myofibrillar function, and performed running wheel tests.

MATERIALS AND METHODS

Transgenic animals

Studies were performed on 12-week-old female $\beta_{1/2/3}$-AR-KO mice (TKO = $\beta_{1/2/3}$-KO = $\beta_1^{-/-}\beta_2^{-/-}\beta_3^{-/-}$) compared to the respective wildtype mice (WT = $\beta_1^{+/+}\beta_2^{+/+}\beta_3^{+/+}$). Creation of the TKO-mice has been described before (Jimenez *et al.* 2002). Mice were maintained at an artificial light/dark cycle at 20-22 °C with free access to water and standard laboratory chow diet. Following cervical dislocation the hearts were immediately taken out for further experiments and prepared as described below. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985). The experiments were approved by the local animal ethics committee.

Morphological analysis

After being weighed hearts were fixed with 4% paraformaldehyde, dehydrated with alcohol, embedded in paraffin, and cut into 7 µm slices, which were stained with hematoxylin/eosin. For each group 4 slices of the left anterior wall were obtained and 5 photographs were taken at 400fold magnification (Leica DC500) respectively.

Cross-sectional areas of 5 cardiomyocytes were determined by digitizing the images and computerized pixel counting including only nucleated myocytes for the analysis as described by Engelhardt et al. (Engelhardt *et al.* 1999).

Echocardiography

Transthoracical echocardiography was performed in 3-month-old mice as previously described (Tiemann *et al.* 2003). Mice were anesthetized with enflurane (3% for induction and 0.8–1% for echocardiography) in 50% nitrous oxide and 50% oxygen by face mask. The degree of anesthesia was adapted to minimize cardiodepressive effects. Thus the heart rate during anesthesia was maintained in the physiological range of conscious mice under resting conditions. The core temperature was maintained at 37°C using a feedback-controlled heating pad. Echocardiography was performed using a commercially available ultrasound system equipped with a linear array transducer operating at an emission frequency of 15 MHz (harmonic-mode) with frame rates up to 280 Hz (HDI-5000, Philips Medical Systems, Bothell, WA, USA). Analysis included heart rate, left ventricular end-systolic and end-diastolic volume. For calculation of volumes the area length method was used (Ghanem *et al.* 2006, Tiemann *et al.* 2003).

Tissue preparation

For the preparation of Western blot samples tissue of each heart was prepared as previously described (Munch *et al.* 1998). Briefly, 25-35 mg of left ventricular myocardial samples were thawed on ice in threefold volume of chilled preparation buffer (in mmol/l: sucrose 300, phenyl methyl sulfonyl fluoride 1, piperazine-N,N-bis(2-ethane sulfonic acid) 20, EDTA 1, P_i 10, pH 7.4). Care was taken to dissect

myocardial tissue from connecting tissue, vessels, and pericardium. Samples were homogenized with a glass-teflon homogenizer (Potter Typ RN 24, 350 upm, IKA Labortechnik Stauffen) under constant 4 °C temperature. The homogenates were immediately shock-frozen in liquid nitrogen and stored at – 80 °C in freezing buffer (in mmol/l: sucrose 400, NaH_2PO_4 50, EDTA 10, piperazine-N,N-bis(2-ethane sulfonic acid) 5, 2-amino-2-(hydroxymethyle)-1.3.propandiol 5, pH 7.2). Protein content was measured according to Bradford (Bradford 1976).

Western blot analysis

For detection of SERCA 2a, PLB, phosphorylated PLB-Ser16, phosphorylated PLB-Thr17, ryanodine receptor and Na^+/Ca^{2+}-exchanger (NCX) in WT and TKO myocardium, immunoblotting techniques were performed as described previously, with slight modifications (Schwinger *et al.* 1995). Equal amounts of membrane proteins (10 µg) were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) with 4% stacking gel and 12% acrylamide. The linear range of the antibodies used was determined in previous experiments (Schwinger *et al.* 1995). To control protein load, we performed Coomassie blue stainings of the gel. After blotting, we controlled protein transfer using Ponceau red staining of the membrane. For the immunoreaction, the following commercially available primary antibodies were used: anti-SERCA 2a (mouse) from Affinity BioReagents (Golden, CO, USA); anti-ryanodine receptor (mouse) from Affinity BioReagents; anti-PLB phosphorylated at Ser16 and Thr17 (rabbit), antibodies against sequence RSAIRRASTIEY (residues 9-19 of PLB, +Y) in which Ser16 or Thr17 is phosphorylated, from Cyclacel (Dundee, UK); anti-PLB (mouse) from Upstate Biotechnology (Dundee, UK), clone A1, anti-Na(+)/Ca(2+) exchanger (mouse) from

Novus Biologicals (Littleton, CO, USA). For detection, after washing procedures with TBST and TBS, a secondary peroxidase-conjugated mouse or rabbit IgG (Sigma) was used where appropriate. To detect antibody binding, an enhanced chemoluminescence assay (Lumi-Light Western Blotting Substrate, Roche, Grenzach, Germany) was used. After exposure to film (Hyperfilm ECL, Amersham Biosciences, Buckinghamshire, UK) and scanning, the bands were quantified by densitometry.

Preparation of the SR

The SR was prepared according to the methods described by Meissner and Henderson (Meissner and Henderson 1987) and Sitsapesan and Williams (Sitsapesan and Williams 1990). Myocardial tissue was ground in liquid nitrogen. The following preparation was made at 4 °C in the cold room. The tissue was chilled in ice-cold homogenisation buffer (in mmol/l: sucrose 300, PMSF 1, PIPES 20, EDTA 10 and NaH_2PO_4 50, pH 7.4), containing NaH_2PO_4 to inhibit phosphatases, thereby avoiding in vitro PLB dephosphorylation. PMSF irreversibly inhibits serine proteases by sulfonylation of the serine residue in the active site of the protease and thereby avoids protein decomposition. Connective tissue was carefully trimmed away, and myocardial tissue was homogenized with a motor-driven homogenizer (Braun, Melsungen, Germany). The preparations were stored at − 80 °C in a buffer containing (in mmol/l) sucrose 400, HEPES 5, Tris 5, EDTA 10 and NaH_2PO_4 50, pH 7.2.

Measurement of SERCA 2a activity

Analysis of SERCA 2a activity was carried out according to the method described by

Chu et al. (Chu *et al.* 1988), which is based on coupled enzymatic reactions as follows: 1. ATP → ADP + P_i (this reaction is catalysed by SERCA 2a), 2. ADP + phosphoenolpyruvate → ATP + pyruvate (catalysed by pyruvate kinase), 3. pyruvate + NADH → lactate + NAD^+ (catalysed by lactate dehydrogenase). The oxidation of NADH was continuously monitored by the decrease in absorbance at 340 nm with a spectrophotometer (Beckman DU 640). The reaction was carried out in 1 ml at 37 °C. 20 µl of the SR preparation (final protein concentration, 50 µg/ml) was suspended in 1000 µl of the reaction mixture. The reaction mixture had the following composition (in mmol/l: MOPS 21, NaN_3 4.9, EGTA 0.06, KCl 100, $MgCl_2$ 3, phosphoenolpyruvate 1, NADH 0.2 and pyruvate-lactate dehydrogenase LDH enzyme mixture (8.4/12 U)). This led to the following concentrations in the cuvette (in mmol/l): MOPS 21, NaN_3 4.9, EGTA 0.06, KCl 100, $MgCl_2$ 3, phosphoenolpyruvate 1, NADH 0.2, sucrose 7, HEPES 0.05, Tris 0.05, EDTA 0.2, PMSF 0.01, PIPES 0.2 and NaH_2PO_4 1, pH 7.2. $CaCl_2$ was added to the reaction mixture to yield the desired free Ca^{2+} concentrations, calculated according to the method of Fabiato and Fabiato (Fabiato and Fabiato 1979). Calculations performed by this method are an approximation. We did not measure Ca^{2+} concentrations directly. The reaction was started with ATP (1 mmol/l) and was constant over at least 5 min. The basal activity was measured in the absence of Ca^{2+} and in the presence of EGTA (4 mmol/l) simultaneously. All experiments were carried out in duplicate. Experiments were performed as described before (Schwinger *et al.* 1995).

Triton X skinned fibres measurements

Left ventricular muscle fibres were prepared as previously described (Brixius and Schwinger 2000). Briefly, the fibre bundles (diameter < 0.2 mm) were dissected from

the left ventricular papillary muscles and permeabilised at 4 °C for 20 h in a solution containing 50% (v/v) glycerol, 1% Triton X, and (in mmol/l) NaN_3 10, ATP 5, $MgCl_2$ 5, EGTA 4, 1,4-dithioerythritol (DTE) 2, and imidazole 20 (pH 7.0). Afterwards, the fibres were stored in a similar solution but without Triton X at − 20 °C. For the experiments, the Triton X skinned fibre bundles were prepared under the microscope and then mounted isometrically and connected to a force transducer in a perfusion cuvette (Scientific Instruments, Heidelberg, Germany) in relaxation solution (composition in mmol/l: imidazole 20, Na_2ATP 10, NaN_3 5, EGTA 5, $MgCl_2$ 12.5, dithiothreitol 1, creatine phosphate 10, creatine kinase 1 mg/ml, free Ca^{2+}: 10 nM, pH 7.0). Fibre length was adjusted so that resting tension was just threshold (slack position). The fibres were then incubated in contraction solution (composition as above, but with increasing concentrations of free Ca^{2+} estimated according to the method of Fabiato and Fabiato (Fabiato and Fabiato 1979)). The experiments were performed at room temperature. To normalize force, the absolute force values obtained were divided by the cross-sectional area of the skinned fibres (CSA = 2πr2). Experiments were performed as described previously (Brixius and Schwinger 2000).

Measurement of force development

Force development was measured as described before (Brixius and Schwinger 2000, Guth and Wojciechowski 1986) (experimental setup, Scientific Instruments, Heidelberg, Germany). The relaxation solution contained (in mmol/l) imidazole 20, Na_2ATP 10, NaN_3 5, EGTA 5, $MgCl_2$ 12.5, phospho(enol)-pyruvate 5, NADH 0.6, P_1,P_5-di(adenosine 5') pentaphosphate 0.2 (myokinase inhibitor), and cyclopiazonic acid 25, together with pyruvate kinase 100 U/ml and lactate dehydrogenase 125 U/ml. The contraction solution contained calcium EGTA 5 mmol/l instead of EGTA. Both

solutions were mixed by a gradient mixer so that Ca^{2+} was successively increased every 30 s. Free Ca^{2+} concentration was determined by calculator programs designed for experiments in skinned muscle cells (Fabiato and Fabiato 1979). Measurement of developed tension started 3 s after the solution was exchanged. Developed tension had reached a stable plateau at that time.

Running wheel tests

Running wheel tests have been shown to be suitable to assess cardiovascular function of transgene mouse models of human cardiovascular diseases (Bernstein 2003). Mice were held in cages for 4 days in quiet surroundings at room temperature of 20-22 °C having the possiblity to exercise on running wheels with free access to water and standard laboratory chow diet. The results were measured by a tachometer attached to the running wheel.

Materials

All chemicals were of analytical grade or the best grade commercially available. All compounds were dissolved in twice-distilled water and did not change the pH of the medium.

Statistical analysis

Data are presented as mean ± standard deviation. Data analysis was performed using Student's t test for paired and unpaired data, where appropriate. Significance was considered at a p-value < 0.05.

RESULTS

Morphometric analysis and echocardiography

Body weight was similar in TKO- and WT-mice, whereas heart weight and heart-to-body-weight ratio were significantly decreased in TKO. In line with these findings, myocyte cross-sectional area was also significantly decreased. Echocardiographic analysis provided evidence that heart rate and ejection fraction were significantly diminished in TKO as compared to WT. Thus, chronic β-AR deficiency is accompanied by cardiac hypotrophy as well as negative inotropy and chronotropy (tab. 1).

Ca^{2+}-regulatory proteins

To investigate whether the negative inotropy may be due to alterations of the Ca^{2+}-regulatory proteins, we performed Western blot experiments comparing TKO (n = 5) and WT (n = 4). The expression of the ryanodine receptor, the Na^{+}/Ca^{2+}-exchanger and of SERCA 2a were unchanged in the TKO compared to WT. The expression of phospholamban was significantly increased, whereas phosphorylation of phospholamban at serine 16 and threonine 17 revealed a marked decrease (fig. 1). In line with these findings, maximal SERCA 2a activity was significantly decreased in TKO compared to the WT (fig. 2).

Myofibrillar function

To assess myofibrillar function, we measured Ca^{2+} sensitivity of skinned fibres in TKO and WT mice by performing measurements of force development of Triton X skinned fibres within increasing Ca^{2+} concentrations (fig. 3). A similar maximal calcium-dependent tension (WT 22.6 ± 7.7 mN/mm², n = 6; TKO 26.9 ± 13.4

mN/mm², n = 6; n.s.), but an increased myofibrillar calcium-sensitivity was measured in TKO as compared to WT (EC_{50}: WT 1.49, 95% confidence interval 1.36-1.62 µM, n = 6; TKO 0.97, 95% confidence interval 0.83-1.11 µM, n = 6).

Running wheel tests

To investigate the influence of the cardiac alterations observed in TKO mice on the exercise capacity, TKO and WT mice performed a voluntary running wheel experiment. Mean velocity (WT 0.34 ± 0.33 km/h, n = 6; TKO 0.81 ± 0.44, n = 6), trip distance (WT 0.33 ± 0.60 km/d, n = 6; TKO 1.19 ± 0.72 km/d, n = 6) and trip time (WT 29.0 ± 37.7 min/d, n = 6; TKO 86.6 ± 46.5 min/d, n = 6) were significantly increased in TKO as compared to WT (fig. 4).

DISCUSSION

The present study investigated the influence of β-adrenoceptor deficiency on cardiac function using a knockout model. It was shown that the $\beta_{1/2/3}$-KO leads to a significantly decreased heart weight, heart rate and ejection fraction accompanied by altered expression and activity of important Ca^{2+}-regulating proteins. Chronic $\beta_{1/2/3}$-AR deficiency reduced SERCA 2a activity by an increase in the expression level of PLB and a diminished PLB-Ser16 and PLB-Thr17 phosphorylation. In spite of these findings, we did not observe relevant functional impairments in running wheel tests but rather an increased willingness to run. The decreased SERCA 2a activity following β-adrenoceptor deficiency may be, at least partly, compensated by an increased myofibrillar calcium-sensitivity.

The regulation of cardiomyocyte growth under physiological and pathophysiological conditions is very complex, but one of its controlling signalling pathways is the β-adrenergic system (MacLellan and Schneider 2000). In β_1-overexpression (OE)-mice, an increased heart-to-body weight ratio was described which found its expression in a substantial hypertrophy of cardiomyocytes and a concomitant fibrosis (Engelhardt *et al.* 1999). While in early studies β_2-OE-animals did not seem to have any morphological differences compared to their WT, it was shown in later studies that overexpression of the β_2-adrenoceptor was linked with cardiac hypertrophy associated with a cumulative mortality of 81% within 15 months compared to 4% in WT-mice (Du *et al.* 2000). β_3-OE-mice featured a decreased heart-to-body weight ratio compared to their WT (Kohout *et al.* 2001). Whereas overexpression of the various β-adrenoceptor subtypes alters cardiac morphology, neither the β_1- nor the β_3-KO-mice had an altered heart-to-body weight ratio (Rohrer *et al.* 1996, Varghese *et al.* 2000). Recently it could be shown, however, that a $\beta_{1/2}$-knockout leads to a decreased heart-to-body weight ratio (Kiriazis *et al.* 2008). Since the present study also found a decrease in the heart-to-body weight ratio in TKO-mice, whereas the β_1- and β_3-knockouts did not effect the heart-to-body weight ratio at all, it may be concluded that in maintaining a certain myocyte volume there has to be a functional redundancy among the β-adrenoceptors, so that only a blockade of at least the β_1- and the β_2-adrenoceptor entails significant changes in the heart-to-body-weight ratio.

Human heart failure is characterized by profound dysregulation of intracellular Ca^{2+}-homeostasis. Thus, structural and functional alterations in the Ca^{2+} regulatory proteins present in the sarcoplasmic reticulum (SR) have been shown to be strongly involved in the pathogenesis of heart failure (Yano *et al.* 2005). Although it is a matter

of an ongoing discussion whether SERCA 2a and PLB expression is altered, it can be stated that SERCA 2a activity is depressed in the setting of human heart failure due to an attenuated PLB-Ser16- and PLB-Thr17 phosphorylation (Dash *et al.* 2001, Schwinger *et al.* 1995). PLB phosphorylation causes a disinhibition of SERCA 2a resulting in an enhanced diastolic Ca^{2+} sequestration into the SR (Frank *et al.* 2003).

The diminished SERCA 2a activity we found in TKO-mice was due to an increased PLB expression and a decreased PLB-Ser16 and PLB-Thr17 phosphorylation. It should be pointed out, that PLB-Thr17 phosphorylation was decreased, yet not totally absent in $\beta_{1/2/3}$-deficient mice. For some time it has been discussed whether Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII)-dependent phosphorylation of PLB-Thr17 is reliant on the presence of β-adrenergic stimulation or not. Whereas some studies came to the conclusion that phosphorylation of PLB-Ser16 and β-adrenergic stimulation respectively are prerequisites for PLB-Thr17 phosphorylation, other studies reported PLB-Ser16 independent phosphorylation of PLB-Thr17. [reviewed in (Mattiazzi *et al.* 2005)] Finally experiments with a phosphatase inhibitor ('okadaic acid') showed, that PLB-Thr17 can indeed be phosphorylated in the absence of significantly elevated cAMP-levels or concomitant PLB-Ser16 phosphorylation (Mundina-Weilenmann *et al.* 1996). Regarding the β-adrenoceptor independent phosphorylation of phospholamban, the important role of type 1 phosphatase (PP1) has to be mentioned. PP1 represents the main SR phosphatase which among others dephosphorylates CaMKII. This facilitates CaMKII to perpetuate its activity independently of the intracellular Ca^{2+}-concentration (Bradshaw *et al.* 2003). It may be speculated that this possibility of compensation eluding the β-

adrenergic system may be responsible for the slightly sustained PLB-Thr17 phosphorylation in TKO-mice. Further studies have to verify that assumption.

It is somewhat surprising that a complex organism, that a mouse represents, can survive without any β-adrenoceptors. Obviously the organism has other mechanisms besides the β-adrenergic system with which cardiovascular function can be sustained. One of these mechansims might be the increased myofibrillar calcium-sensitivity which could be found in TKO-mice. Furthermore, studies with β_1-KO-mice could show, that heart rate in these animals is regulated by the vagal tone, therefore by the parasympathetic nervous system (Rohrer *et al.* 1998). In addition, it has been shown that TKO-mice have a reduced metabolic rate compared to WT (Bachman *et al.* 2002). This can be seen as a contributing factor to the reduced cardiac output found in these animals. So despite of a complete KO of the β-adrenoceptors the TKO-mice still performed even better in the running wheel tests than their WT littermates. Whereas both β_1- and $\beta_{1/2}$-KO-mice showed no differences in exercise capacity in treadmill experiments (Rohrer *et al.* 1999, Rohrer *et al.* 1998), β_2-KO-mice also presented with an increased exercise capacity (Chruscinski *et al.* 1999). In these mice during exercise fat burning was increased which led to a prolonged availability of glycogene reserves in the musculature while the body fat content was significantly lower in β_2-KO-mice compared to WT-mice. So it seems that the greater exercise capacity of TKO-mice may be, at least partly, attributed to the lack of the β_2-AR. A limitation of the present study is that animals were running voluntarily. Therefore, it is possible that the presented data do not necessarily represent maximal exercise capacity of these animals.

CONCLUSION

Despite β-adrenoceptor deficiency is going along with cardiac hypotrophy and a decrease in cardiac function, willingness to run is not impaired. These results indicate that compensatory mechanisms may be activated to maintan cardiac function in increased cardiac stress situations. These mechanisms have to be focussed in future studies.

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Disclosures

No potential confilct of interest relevant to this article was reported.

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TABLE

Table 1: *Morphometric analysis and echocardiography*

Parameter	WT	TKO	p-value
Body weight [g]	30.81 ± 3.08 (n=5)	31.00 ± 0.94 g (n=5)	0.900
Heart weight [mg]	121 ± 3 (n=5)	94 ± 7 (n=5)	< 0.001
Heart to body weight ratio [mg/g]	3.96 ± 0.49 (n=5)	3.02 ± 0.18 (n=5)	0.004
Cross sectional area [% of WT]	100 ± 26.3 (n=4)	67.0 ± 31.6 (n=5)	< 0.001
Heart rate [beats/min]	403 ± 24 (n=4)	267 ± 69 (n=7)	0.005
Left ventricular end-systolic volume [µl]	7.4 ± 4.9 (n=4)	15.1 ± 5.0 (n=7)	0.036
Left ventricular end-diastolic volume [µl]	37.2 ± 6.1 (n=4)	39.0 ± 6.2 (n=7)	0.649
Ejection fraction	81 ± 11 (n=4)	62 ± 9 (n=7)	0.013

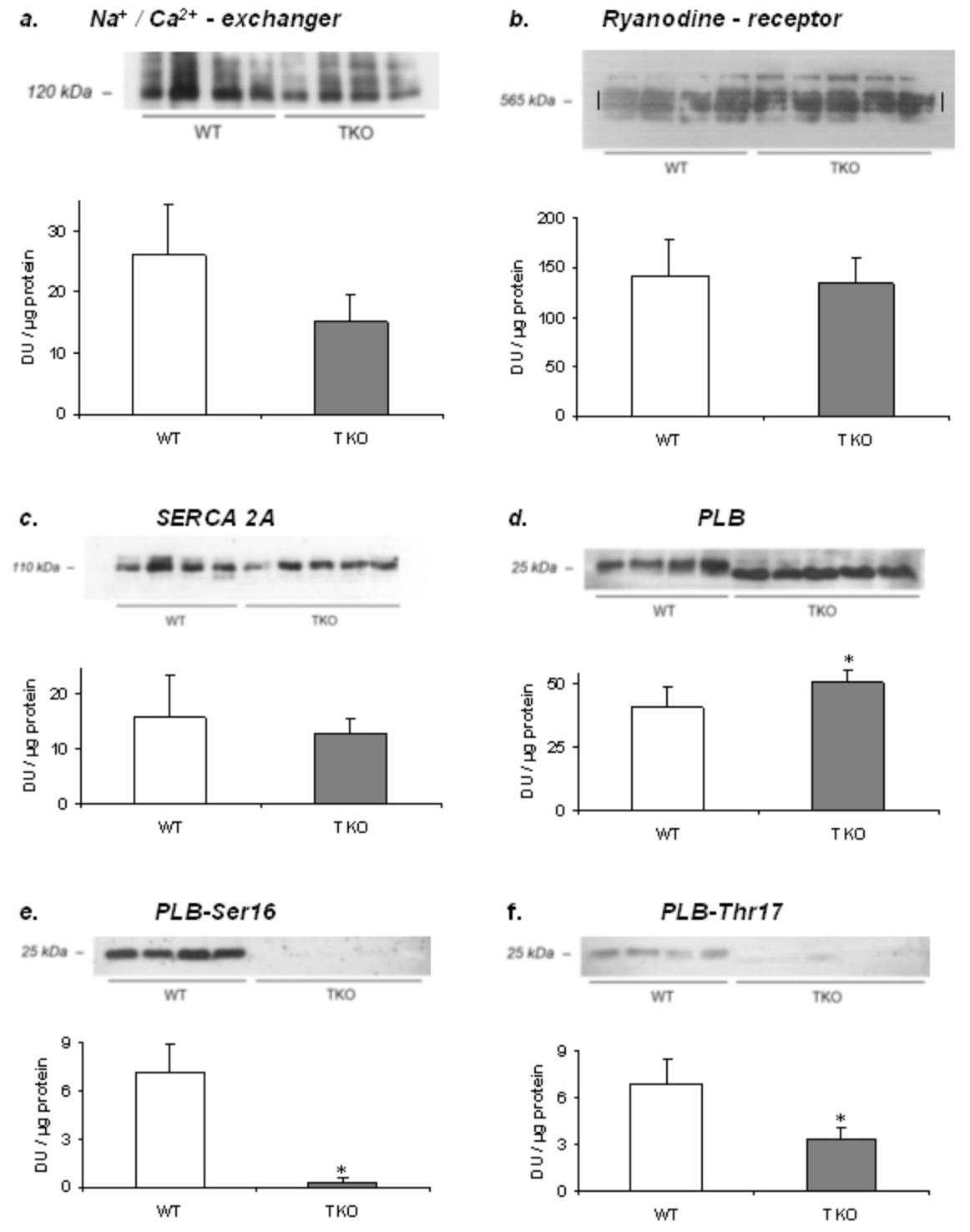
FIGURE LEGENDS

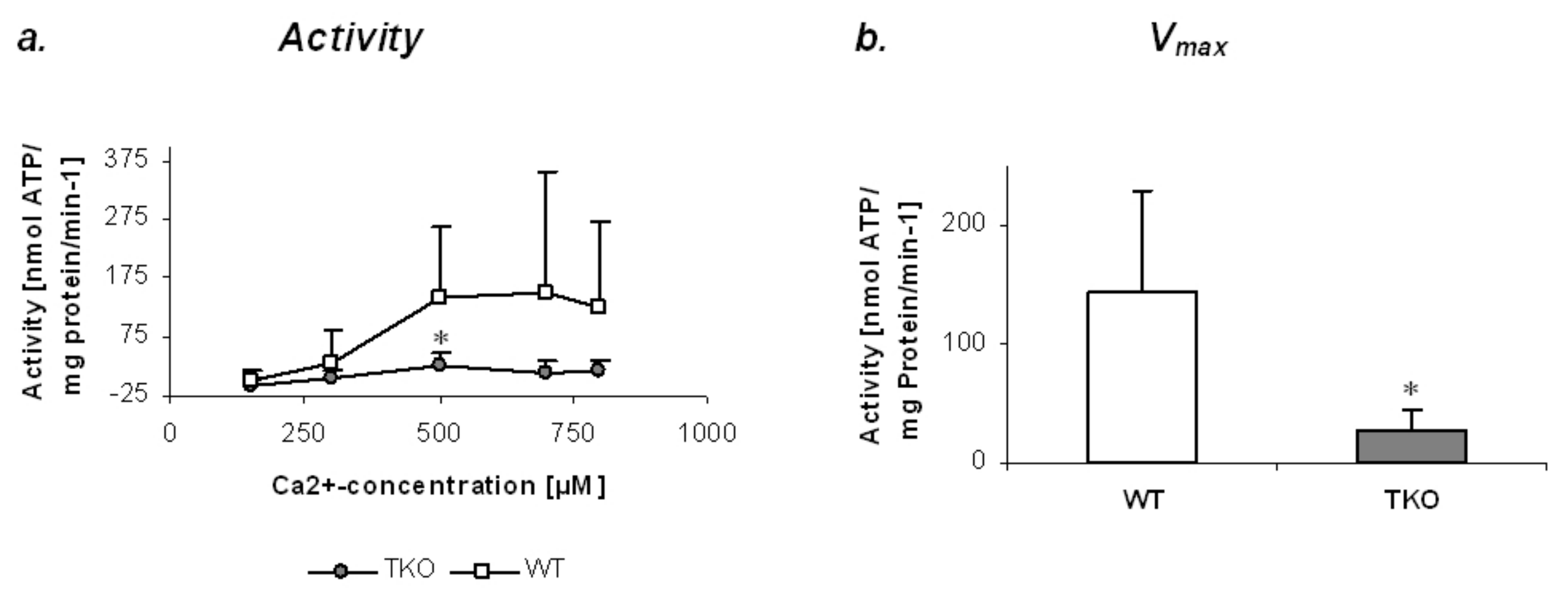
Figure 1: The protein expression of NCX, ryanodine receptor and SERCA 2a was not significantly changed, whereas PLB (∗ : $p < 0.02$ vs. WT) expression was significantly increased and PLB-Ser16 (∗ : $p < 0.0001$ vs. WT) and PLB-Ser17 (∗ : $p < 0.005$ vs. WT) expression was decreased in TKO-mice. Data was normalized to Calsequestrin expression.

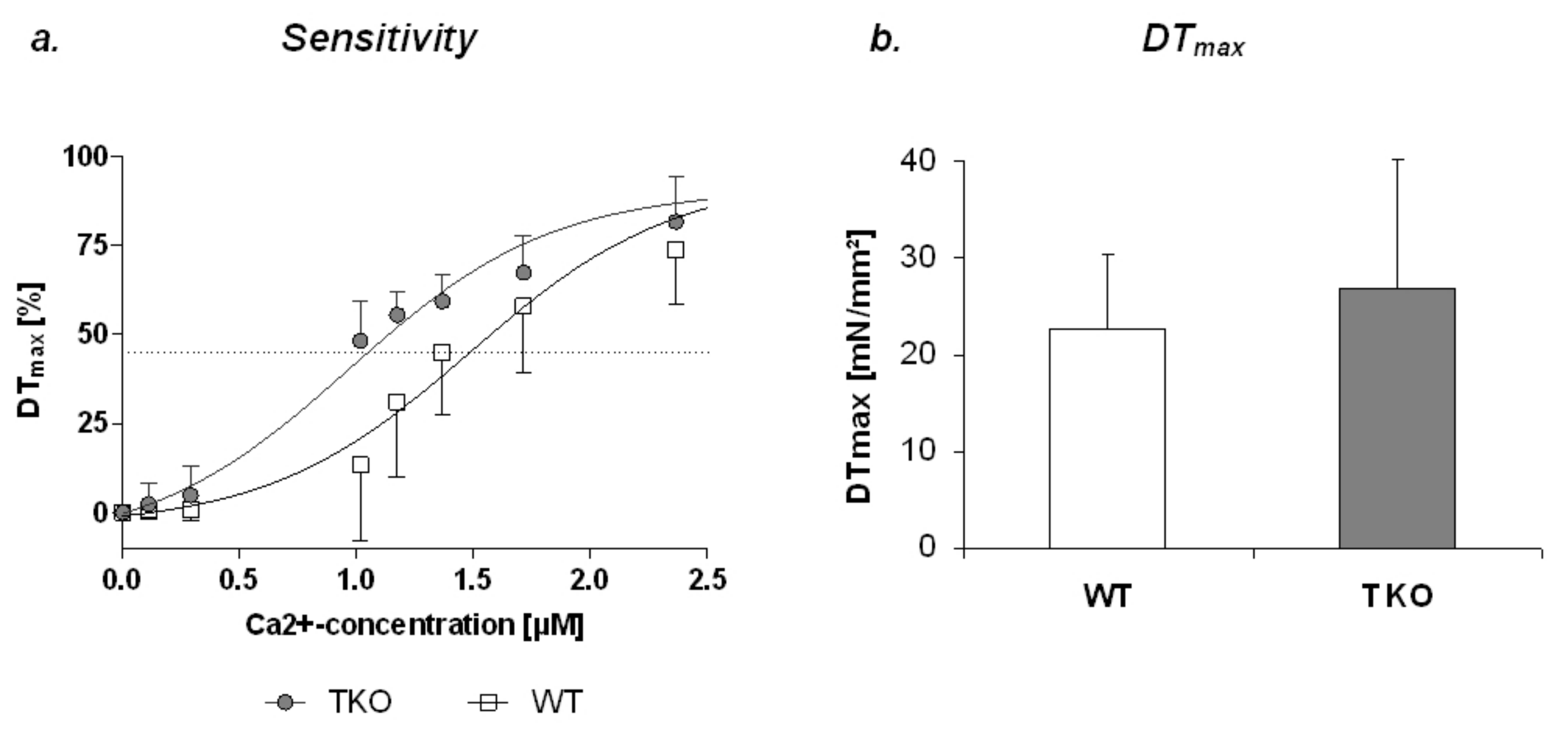
Figure 2: The maximal activity (V_{max}) of SERCA 2a in TKO-mice was 28.1 ± 16.3 nmol ATP/mg protein/min^{-1} (n = 6) compared to 144.4 ± 84.0 nmol ATP/mg protein/min^{-1} (n = 4) in WT-mice. Thus the activity of SERCA 2a was significantly decreased. (∗ : $p < 0.01$ vs. WT)

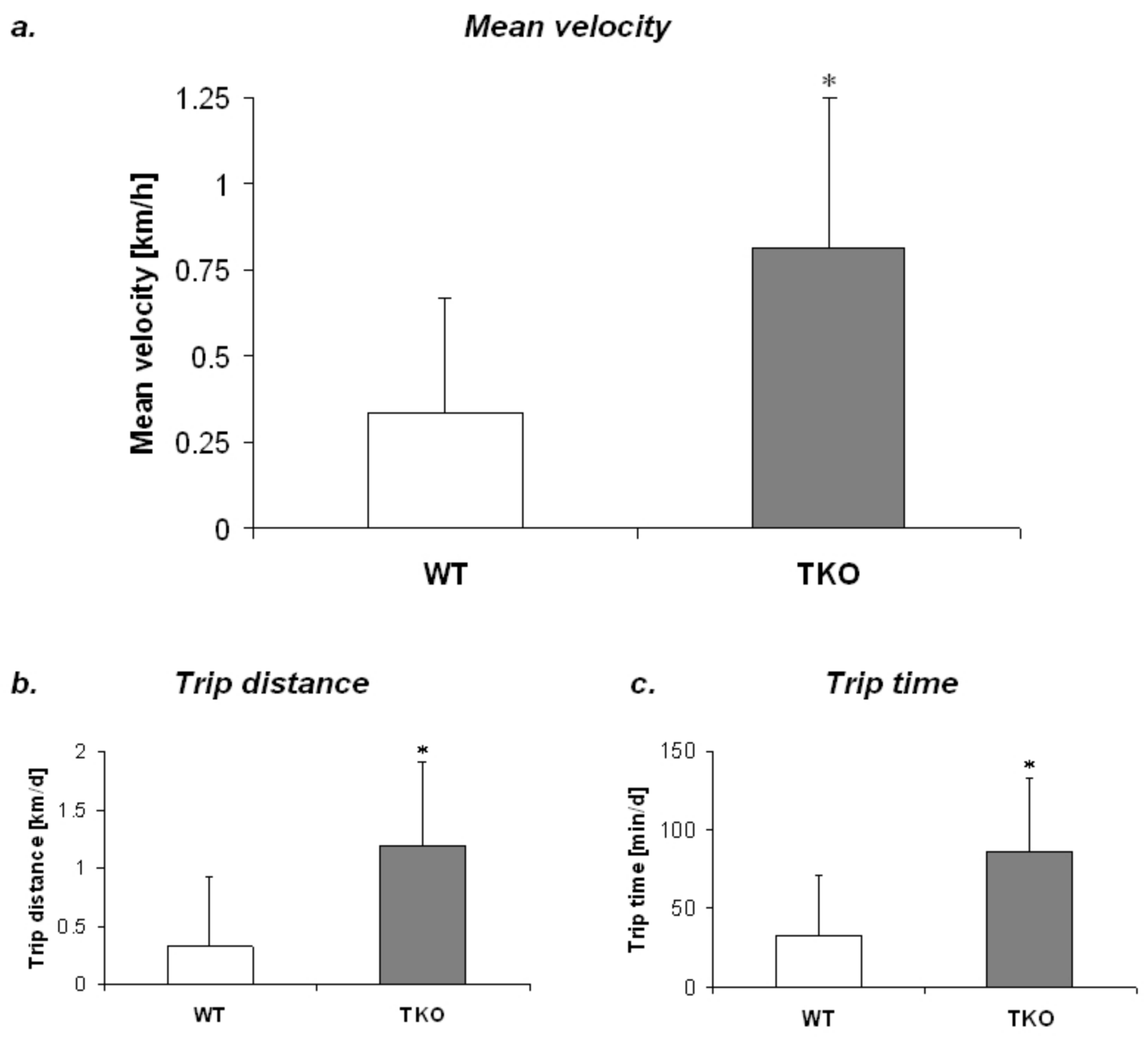
Figure 3: Whereas the maximal force (DT_{max}) of skinned fibers in TKO mice was similar to WT mice, calcium-sensitivity was significantly increased in TKO mice compared to WT mice.

Figure 4: In running wheel tests there could not be shown any diminished exercise capacity of TKO mice compared to the WT. Rather an increased willingness to run could be observed which reached statistical significance for mean velocity (∗ : $p < 0.04$ vs. WT), trip distance (∗ : $p < 0.03$ vs. WT) and trip time. (∗ : $p < 0.04$ vs. WT)


a.
Na+ / Ca2+ - exchanger
120 kDa
WT
TKO
DU / µg protein
0
10
20
30
WT
TKO
b.
Ryanodine - receptor
565 kDa
WT
TKO
DU / µg protein
0
50
100
150
200
WT
TKO
c.
SERCA 2A
110 kDa
WT
TKO
DU / µg protein
0
10
20
WT
TKO
d.
PLB
25 kDa
WT
TKO
DU / µg protein
0
25
50
*
WT
TKO
e.
PLB-Ser16
25 kDa
WT
TKO
DU / µg protein
0
3
6
9
*
WT
TKO
f.
PLB-Thr17
25 kDa
WT
TKO
DU / µg protein
0
3
6
9
*
WT
TKO


a.
Activity
Activity [nmol ATP/ mg protein/min-1]
375
275
175
75
-25
0
250
500
750
1000
Ca2+-concentration [µM]
*
TKO
WT
b.
V_{max}
Activity [nmol ATP/ mg Protein/min-1]
200
100
0
*
WT
TKO


a.
Sensitivity
100
75
50
25
0
DTmax [%]
0.0
0.5
1.0
1.5
2.0
2.5
Ca2+-concentration [µM]
TKO
WT
b.
DTmax
40
30
20
10
0
DTmax [mN/mm²]
WT
TKO


a.
Mean velocity
Mean velocity [km/h]
1.25
1
0.75
0.5
0.25
0
*
WT
TKO
b.
Trip distance
Trip distance [km/d]
2
1.5
1
0.5
0
*
WT
TKO
c.
Trip time
Trip time [min/d]
150
100
50
0
*
WT
TKO