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Proarrhythmia in a non-failing murine model of cardiac-specific Na^+/Ca^{2+} exchanger overexpression: whole heart and cellular mechanisms

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Abstract

The cardiac Na^+/Ca^{2+} exchanger (NCX) generates an inward electrical current during SR-Ca^{2+} release, thus possibly promoting afterdepolarizations of the action potential (AP). We used transgenic mice 12.5 weeks or younger with cardiomyocyte-directed overexpression of NCX (NCX-Tg) to study the proarrhythmic potential and mechanisms of enhanced NCX activity. NCX-Tg exhibited normal echocardiographic left ventricular function and heart/body weight ratio, while the QT interval was prolonged in surface ECG recordings. Langendorff-perfused NCX-Tg, but not wild-type (WT) hearts, developed ventricular tachycardia. APs and ionic currents were measured in isolated cardiomyocytes. Cell capacitance was unaltered between groups. APs were prolonged in NCX-Tg versus WT myocytes along with voltage-activated K^+ currents (K_v) not being reduced but even increased in amplitude. During abrupt changes in pacing cycle length, early afterdepolarizations (EADs) were frequently recorded in NCX-Tg but not in WT myocytes. Next to EADs, delayed afterdepolarizations (DAD) triggering spontaneous APs (sAPs) occurred in NCX-Tg but not in WT myocytes. To test whether sAPs were associated with spontaneous Ca^{2+} release (sCR), Ca^{2+} transients were recorded. Despite the absence of sAPs in WT, sCR was observed in myocytes of both genotypes suggesting a facilitated translation of sCR into DADs in NCX-Tg. Moreover, sCR was more frequent in NCX-Tg as compared to WT. Myocardial protein levels of Ca^{2+}-handling proteins were not different between groups except the ryanodine receptor (RyR), which was increased in NCX-Tg versus WT. We conclude that NCX overexpression is proarrhythmic in a non-failing environment even in the absence of reduced K_V. The underlying mechanisms are: (1) occurrence of EADs due to delayed repolarization; (2) facilitated translation from sCR into DADs; (3) proneness to sCR possibly caused by altered Ca^{2+} handling and/or increased RyR expression.

Keywords

Na^+/Ca^{2+} exchanger; Cellular electrophysiology; Transgenic mice; Arrhythmia; Ventricular tachycardia; Sudden cardiac death

Introduction

The Na^+/Ca^{2+} exchanger (NCX) is the main Ca^{2+} removal mechanism of the cardiac myocyte. By extruding 1 Ca^{2+} ion in exchange for 3 Na^+ ions, NCX is electrogenic and generates an inward current during Ca^{2+} release from the sarcoplasmic reticulum (SR) (for review see [42]). Via this mechanism, NCX may prolong the action potential (AP) and mediate afterdepolarizations which could trigger cardiac arrhythmia.

There is evidence that NCX activity and expression are upregulated in heart failure [5, 16, 29, 36, 43, 46]. A major cause of death in patients suffering from heart failure is malignant ventricular arrhythmia [20]. However, the mechanisms of sudden cardiac death in heart failure are incompletely understood and it is unknown whether the upregulation of NCX or any other of the many structural or molecular changes associated with heart failure are responsible.

Afterdepolarizations are regarded as the cellular trigger of cardiac arrhythmia. They are defined as oscillations of the electric membrane potential during or just after the cardiac action potential. Early afterdepolarizations (EADs) occur during the plateau or the repolarization phase, while delayed afterdepolarizations (DADs) are observed after full repolarization of the membrane potential (for review see [28]).

The occurrence of EADs is associated with delayed repolarization of the cardiac AP. Thus, the prolonged period of time in depolarized mode will increase the “time window” for the voltage-dependent I_{Ca} and/or I_{Na} to become reactivated and thus trigger an EAD. This has been observed in models of hypertrophy and heart failure where the prolongation of the cardiac AP has been attributed to downregulation of K^+-carried outward currents (for review see [40]). Similarly, loss-of-function mutations of K^+ channel subunits or Na^+ channel gain-of-function mutations [27] can both prolong the AP and cause EADs resulting in clinically relevant ventricular arrhythmia and sudden cardiac death.

We [31, 32] and others [1, 26, 45] have demonstrated that increased NCX activity can also slow repolarization. Thus, potentially, increased NCX activity is a further candidate for mediating EADs via this mechanism.

A similar hypothesis has been suggested to explain the generation of DADs. Thus, the initiating event of a DAD may consist of a spontaneous diastolic release of Ca^{2+} from the SR. This increase in cytosolic Ca^{2+} would then lead to a sudden activation of NCX inward current (I_{NCX}), which would depolarize the sarcolemmal membrane to a potential from which a new and premature AP is triggered [7, 9, 52]. Initial studies using isolated cardiac myocytes from different species have demonstrated that afterdepolarizations can indeed be triggered by spontaneous Ca^{2+} release from the SR [11, 18, 41, 48].

Further studies using animal models of heart failure and hypertrophy have demonstrated that the upregulation of NCX expression may also play a role in the generation of DADs [5, 12, 22, 29, 30, 43, 49]. Although in the failing heart, SR Ca^{2+} load is reduced, other factors, for example increased ryanodine receptor (RyR) activity due to increased phosphorylation, lead to an increased rate of spontaneous SR Ca^{2+} release in heart failure [2, 28].

Yet, in human and animal heart failure, a multitude of structural, expressional and functional changes other than the upregulation of NCX have been observed ([23, 44], for review see [51]). Most importantly, a reduction of the repolarizing K^+-carried outward current has been held responsible for the delay in repolarization and proarrhythmia in heart failure [6, 28].

It thus remains unclear whether increased NCX activity is an independent promoter of cardiac arrhythmia, i.e., whether proarrhythmia occurs as a consequence of specific NCX overexpression or secondary to one or a combination of the several other changes occurring in heart failure. This question carries important implications for the development of novel pharmacological or genetic therapies. Here, we used whole heart preparations and isolated cardiac myocytes from transgenic mice with cardiomyocyte-specific 3.1-fold homozygous overexpression of NCX, which at that age did not show signs of LV dysfunction. The aim of this study was to investigate whether an isolated over-expression of NCX in a non-failing

environment has the potential to cause proarrhythmia and, if so, which cellular and molecular mechanisms were involved.

Methods

Generation of transgenic mice

Homozygous transgenic NCX overexpressor mice were generated as reported previously [37]. Genotyping was performed using PCR. All experiments were performed in accordance with the University of Münster's regulation on animal research.

Echocardiography and surface ECG

Echocardiographic and surface ECG data were recorded and analyzed as previously reported [13].

Western blots

Thirty-five milligrams of frozen murine ventricles were homogenized at 4°C for 3 min in 0.9 mL of a medium containing (in mM/L) 10 $NaHCO_3$, 50 NaF, 5 $Na_4P_2O_7$ and 20% SDS (pH 7.4), or a medium containing 20% SDS, 10 mM/L $NaHCO_3$ using a Polytron (PT-3000) homogenizer (Kinematica AG, Lucerne, Switzerland) and a Vir-Sonic (VirTis Gardiner, NY) as described previously [19]. For immunoblot analysis, 200 μg of the homogenate was used for blotting NCX, 25 μg for troponin I (Tni), junctin (Jcn) and triadin (Trd), 50 μg for phospholamban (Plb), calsequestrin (CsQ) and SERCA, and 250 μg for RyR and phosphorylated RyR (RyRp). The homogenates were electrophoretically separated on SDS-polyacrylamide gels. After transferring the proteins to nitrocellulose, the blots were incubated with specific antibodies aimed against the following proteins: NCX (R3F1 SWANT; Bellinzona (Switzerland)), Plb (Upstate, New York, NY, USA), CsQ (Affinity BioReagents; Dianova GmbH Hamburg, Germany), Ser2808 phosphorylated RyR (Badrilla; Leeds; UK), Tni (Cell Signaling Technology, Inc. Danvers, USA), and antibodies against the RyR, SERCA2a, Trd and Jcn were generously provided by Dr. L.R. Jones (Indianapolis, USA). NCX and Tni were detected by alkaline phosphatase-conjugated and horseradish peroxidase-conjugated antibodies. CsQ, RyR, phosphorylated RyR, SERCA, Trd and Jcn were marked by ^{125}I-labeled protein A (Amersham Biosciences). Plb was assayed using the NBT/BCIP substrate kit. The blots were quantified with the help of ImageQuant using a Storm 860 (Molecular Dynamics, Sunnyvale, CA, USA). NCX, SERCA, Jcn, Trd, Plb, Tni and RyR values were normalized by taking a ratio with the housekeeping protein CsQ.

Beating whole heart Langendorff model

Mice were anesthetized by an intraperitoneal injection of urethane (2 g/kg bodyweight). Prior to excision of the heart, a 12-lead ECG was recorded [21]. Thereafter, mice were heparinized and their hearts excised. The hearts were perfused on a modified Langendorff apparatus [21]. A 2.0-French octapolar catheter (interelectrode distance 500 μm) was inserted through the tricuspid orifice into the right ventricle, and two to three custom-made monophasic action potential catheters were placed on the epicardium of the right and left ventricular free wall and the interventicular septum [14]. AV block was induced to facilitate ventricular bradycardia. This intervention was sufficient to provoke repolarization-dependent arrhythmias in susceptible hearts [14]. After observing spontaneous rhythm to identify ventricular arrhythmias, the heart was stimulated from the center of the multielectrode or from the tip of the octapolar catheter at twice diastolic threshold for recording of steady-state monophasic action potentials and to determine effective refractory periods using published techniques [14]. All recordings were preamplified, digitized at 1–2 kHz and analyzed off-line for action potential duration, heart rate, PQ and QT intervals, and

arrhythmias (EMKA, Paris, France). All functional experiments and analyses were performed blinded to genotype.

Isolation of ventricular myocytes from adult mice hearts

Adult mice aged 10–12.5 weeks were pretreated with heparin (5 units/g body weight) 30 min before killing them with carbon dioxide. Hearts were quickly removed via thoracotomy and the cannulated aorta was fixed to a Langendorff perfusion apparatus. Single ventricular myocytes were isolated enzymatically using a collagenase/protease mix according to Mitra and Morad [24] and as applied by us previously [32] with minor modifications.

Cellular electrophysiology

Isolated cardiac myocytes were placed in an experimental chamber (2 mL) mounted on the stage of a Leica DMIL or Nikon Diaphot inverted microscope. A bath solution at room temperature continuously perfused the chamber. Patch electrodes were pulled from borosilicate glass (World Precision Instruments, Sarasota, FL, TW150F-3) on a Narishige Model PC-10 or Sutter P97 horizontal puller to an electrode tip diameter of 2–3 μm. I_{Ca}, I_K and I_{NCX} were recorded in the voltage clamp mode. For current clamp measurements, we used the perforated patch technique. Amphotericin B was added to the patch electrode solution to a final concentration of 260 μg/L [34]. Unless otherwise noted, the patch electrode solution contained (mM/L): 90 KCl, 5 NaCl, 35 KOH, 2.5 MgATP, 1 EGTA, 5 HEPES, pH 7.4 with KOH. The bath solution contained (in mM/L): 136 NaCl, 5.4 KCl, 10 HEPES, 1 $MgCl_2$, 0.33 NaH_2PO_4, 1 $CaCl_2$, 10 Glucose, pH 7.4 with NaOH. An Axopatch 200B patch clamp amplifier (Axon Instruments, Union City, CA, USA) and a Digidata 1200 or 1322A (Axon Instruments) data acquisition system controlled by pCLAMP 9 software (Axon Instruments) were used. We applied series resistance compensation to all current recordings.

Cellular Ca^{2+} imaging

Cells were incubated with 23.3 μM Indo-1 AM for 15 min and then placed in an experimental chamber perfused with bath solution [unless otherwise noted, in mM/L: 2 $CaCl_2$, 140 NaCl, 5.8 KCl, 0.5 KH_2PO_4, 0.4 Na_2HPO_4, 0.9 $MgSO_4$, 11.1 $C_6H_{12}O_6$, 10 HEPES (pH 7.3)]. Myocytes were field stimulated and the emitted fluorescence was recorded at wavelengths of 405 and 495 nm. The ratio of 405/495 nm was taken as a direct measure of Ca^{2+} concentration. We used an Olympus IX50 microscope connected to a dual-emission microfluorescence system (Photon Technologies Inc., South Brunswick, NJ, USA). Data processing was realized by Felix 1.42 software (Photon Technologies Inc., South Brunswick, NJ, USA).

Rapid solution exchange

When caffeine was applied during patch clamp recordings, this was done via rapid solution exchange. Therefore, a multibarrel Perfusion Pencil® (AutoMate Scientific, Inc) was used.

Statistics

Data are expressed as mean ± SEM. Student's unpaired *t* test was used for direct comparisons of WT versus NCX-Tg. The χ^2 test was used for comparison of incidence of cellular or whole heart arrhythmic events in NCX-Tg versus WT. A p value <0.05 was considered to be statistically significant.

Results

Unaltered LV function in NCX-Tg mice

NCX-Tg mice ($n = 9$) exhibited normal cardiac function when compared with WT ($n = 6$) as assessed by echocardiography. NCX-Tg had a non-significant tendency toward increased diastolic posterior and septal wall widths when compared with WT; however, heart weight to body weight ratio was not different between genotypes (Table 1). All animals used for this study were 12.5 weeks or younger. During this period, no differences in mortality were observed in WT versus NCX-Tg.

Expression of calcium-handling and structural proteins in NCX-TG versus WT

Western blot analyses using specific antibodies were conducted to investigate protein expression of key Ca^{2+}-handling and structural proteins (Fig. 1). All proteins except for phosphorylated RyR were normalized to CsQ. Phosphorylated RyR was normalized to total RyR. NCX was overexpressed in NCX-Tg (NCX-Tg: $n = 7$; WT: $n = 5$). The expression of calsequestrin (CsQ), sarcoen-doplasmic reticular Ca^{2+} ATPase (SERCA), junctin, tria-din, phospholamban and troponin I was unaltered in NCX-Tg (NCX-Tg: $n = 6$; WT: $n = 6$). Levels of total and Ser2808 phosphorylated RyR receptors were increased in NCX-Tg when compared with WT (NCX-Tg: $n = 6$; WT: $n = 6$).

Signals of calsequestrin and triadin (not normalized and normalized to each other) were not different between groups; however, there was a non-significant tendency toward lower CsQ signals in NCX-Tg versus WT homogenates. Therefore, to avoid misinterpretations due to normalization, signals of Ca^{2+}-handling proteins were analyzed without normalization, normalized to CsQ and normalized to Trd.

Signals of SERCA, Tni and Plb were not different between groups—not normalized, normalized to CsQ (Fig. 1) and normalized to Trd. Signals of Jcn not normalized as well as JCN signals normalized to CSQ (Fig. 1) were not altered in NCX-Tg versus WT hearts; however, Jcn normalized to Trd was downregulated in NCX-Tg versus WT homogenates.

Signals of total RyR not normalized as well as total RyR signals normalized to CSQ signals were upregulated in TG versus WT hearts; however, there was no difference between groups if RyR signals were normalized to Trd. Taken together, CsQ, Trd, SERCA, Tni and Plb were not significantly different between groups, while Jcn was not regulated or downregulated and total RyR was upregulated or not regulated, subject to the protein used for normalization. Phosphorylated RyR was increased in NCX-Tg when normalized to total RyR.

Prolonged QT interval in NCX-Tg mice

Surface ECGs under resting conditions revealed a prolonged QT interval in NCX-Tg (Fig. 2a), while all other intervals as well as heart rate were not different between genotypes (Table 1).

Proarrhythmia in intact, beating NCX-Tg hearts

To assess the proarrhythmic potential of NCX overexpression, we recorded monophasic action potentials (MAP) in intact beating Langendorff-perfused hearts. MAPs were prolonged at 70 and 90% repolarization in NCX-Tg during pacing at 100 and 150 ms cycle lengths (pacing cycle length 100 ms; APD 70%: WT, 21.0 ± 3.0 ms; $n = 6$; NCX-Tg: 30.9 ± 3.6 ms; $n = 13$; $p < 0.05$). After inducing AV block, 11/16 NCX-Tg hearts spontaneously developed ventricular tachycardias (VT, $n = 8$) and/or premature beats ($n = 3$). None of the five WT hearts developed VT with one exhibiting premature beats (Fig. 2c). The findings in

WT are consistent with previous experiments using this protocol to assess the occurrence of arrhythmia in murine WT hearts [14, 50]. There was a non-significant trend toward APD prolongation at lower frequencies in both genotypes, which was more pronounced in NCX-Tg.

Prolonged action potential duration in NCX-Tg myocytes

Using the patch clamp method, cellular action potentials (AP) were recorded in enzymatically isolated adult myocytes (WT $n = 22$; NCX-Tg $n = 26$; Fig. 3). AP duration at 90% repolarization (APD_{90}) was prolonged in NCX-Tg (perforated patch, room temperature; 1 Hz; NCX-Tg: 295 ± 38 ms; WT: 196 ± 15 ms; $p < 0.05$ vs. WT), while APD_{50}, resting membrane potential and AP amplitude were unaltered between genotypes (resting membrane potential NCX-Tg: −68 ± 0.6 mV; WT: −69 ± 0.7 mV, p [0.05; amplitude NCX-Tg: 108 ± 1.5 mV; WT: 112 ± 1.8 mV, p [0.05).

Increased NCX inward current (I_{NCX}) in NCX-Tg

We recorded I_{NCX} in isolated cardiac myocytes using the patch clamp method in voltage clamp mode (in mM/L, pipette solution: KCl 90; NaCl 5; KOH 35; MgATP 2.5; EGTA 1; HEPES 5; bath solution: NaCl 136; KCl 5.4; HEPES 10; $MgCl_2$ 1; NaH_2PO_4 0.33; $CaCl_2$ 1; glucose 10). Membrane current was recorded during application of 10 mM/L caffeine using a rapid solution exchanger while cells were held at a constant holding potential of −40 mV during exposure to caffeine. To avoid contamination of I_{NCX} with Ca^{2+}-activated I_{Cl}, DIDS (0.1 mM/L) was present. Prior to caffeine application, 15 prepulses were given to ensure steady-state SR Ca^{2+} load. I_{NCX} amplitude was significantly larger in NCX-Tg (normalized to cell capacitance, WT: −1.68 ± 0.14 pA/pF, $n = 10$; NCX-Tg: −4.39 ± 0.57 pA/pF, $n = 15$; $p < 0.05$).

Unaltered sarcoplasmic reticular Ca^{2+} load

Since cytosolic Ca^{2+} is the substrate for NCX inward current and rapid exposure to caffeine induces complete release of Ca^{2+} from the SR, the mathematical integral of $_2{}^+$ content [35, 47]. The integral I_{NCX} is a measure of SR Ca of I_{NCX} was not different in NCX-Tg versus WT (given in arbitrary units normalized to cell capacitance, WT: −0.97 ± 0.1, $n = 10$; NCX-Tg: −1.05 ± 0.11, $n = 15$; $p > 0.05$), thus indicating no difference in SR Ca^{2+} load.

Prolonged L-type Ca^{2+} current activity in NCX-Tg

L-type Ca^{2+} currents (I_{Ca}) were recorded using the patch clamp method. The pipette solution contained (in mM/L): 120 CsCl, 10 TEA-Cl, 10 NaCl, 20 Hepes, 5 MgATP, 0.05 cAMP, pH 7.2 with CsOH. The bath solution contained (in mM/L): 136 NaCl, 5.4 KCl, 10 Hepes, 1.0 $MgCl_2$, 0.33 NaH_2PO_4, 1.0 $CaCl_2$, 10 glucose, pH 7.4 with NaOH. To record L-type Ca^{2+} currents, cells were held at −75 mV and then briefly depolarized to −40 mV to inactivate Na^+ currents. I_{Ca} was then measured during a 200 ms depolarization to 0 mV. Prepulses were given to ensure steady-state Ca^{2+} load of the SR.

We observed no differences in I_{Ca} amplitude in NCX-Tg versus WT (WT: 9.7 ± 0.7 pA/pF; $n = 14$; NCX-Tg: 10.8 ± 0.8 pA/pF; $n = 18$; $p > 0.1$; 0 mV). However, a difference was observed regarding I_{Ca} inactivation kinetics. Thus, in NCX-Tg, I_{Ca} inactivated significantly slower (inactivation constant (τ)—WT: 33.5 ± 1.7 ms; $n = 14$; NCX-Tg: 44.5 ± 2.3 ms, $n = 18$; $p < 0.01$). Parts of these results have previously been published in a congressional proceedings report [31].

Preserved voltage-activated K^+ currents (K_V) and unaltered cell capacitance

Prolongation of cellular AP duration and susceptibility toward arrhythmia have been described to be associated with a reduction of the K^+-carried outward currents [15, 30]. To exclude this possibility, the transient outward current was measured in NCX-Tg versus WT. Cells were held at a resting potential of −80 mV and depolarized to test potentials ranging from −60 to +60 mV (Fig. 4). To avoid contamination of K_v with I_{Na} or I_{Ca}, pipette Na^+ was removed and bath Na^+ was replaced by NMG and nifedipine was added. The patch electrode solution contained (in mM): 140 KCl, 4 Mg-ATP, 1 $MgCl_2$, 10 EGTA, 10 HEPES, pH 7.4 with KOH. The bath solution contained (mM): 130 N-methyl-glucamine (NMG), 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 2 μM nifedipine, 10 glucose, 10 HEPES, pH 7.4 with HCl. Peak K_v was measured by subtracting the steady state from the peak current. K_v was increased in NCX-Tg as compared to WT between test potentials ranging from −30 to +60 mV (ruptured patch, room temperature, +60 mV, normalized to cell capacitance—NCX-Tg: 40.5 ± 6.0 pA/pF; n = 19 cells; WT: 25.4 ± 3.8 pA/pF; n = 20 cells; p <0.05; Fig. 4b).

Before recording K_V, cellular membrane capacitance, which is a measure of cell size and thus an indicator for cellular hypertrophy, was measured. Capacitance was unaltered between NCX-Tg and WT (NCX-Tg: 160 ± 14 pF; n = 19; WT: 177 ± 8 pF; n = 20; p [0.05; Fig. 4b).

Unaltered diastolic Ca^{2+} and systolic Ca^{2+} transient

Ca^{2+} transients and diastolic Ca^{2+} were measured in field-stimulated isolated cardiac myocytes using the Ca^{2+}-sensitive dye indo-1. We did not observe differences in either diastolic (1 Hz; WT: n = 12; NCX-Tg: n = 16; p > 0.05) or systolic Ca^{2+}(1 Hz; Ca^{2+}-transient amplitude given in indo-1 ratio (405/495 nm): WT: 0.090 ± 0,004; n = 12; NCX-Tg: 0.093 ± 0.007, n = 16; p > 0.05).

Increased occurrence of early afterdepolarizations (EAD)

To investigate the cellular mechanisms of proarrhythmia in the face of increased NCX activity, APs were recorded in isolated cardiac myocytes in the current clamp mode. Steady-state pacing was interrupted by sudden changes in stimulation frequency to provoke membrane instability. Therefore, a pacing protocol was chosen with cycle lengths ranging from 1,000 to 8,000 ms (1,000 [1], 2,000 [0.5], 4,000 [0.25], 8,000 [0.125] ms [Hz], steady-state pacing for 20 beats at each cycle length). This was followed by another period of 20 beats each at a cycle length of 8,000 and then 1,000 ms followed by a 3-min period of rest. 22 WT and 26 NCX-Tg myocytes were subjected to this protocol during which membrane potential was continuously recorded.

Early afterdepolarizations (EADs, Fig. 5) were observed in 5 out of 26 NCX-Tg myocytes with an average occurrence of 2.2 ± 0.6 EADs per cell in those cells which exhibited EADs (Fig. 5b). None of the 22 investigated WT cells showed any EAD. When the experiments were repeated in the presence of isoprenaline, the fraction of cells exhibiting one or more EADs (6 out of 22 cells) as well as the average number of EADs registered per cell (6.0 ± 1.7) increased in NCX-Tg (Fig. 5b), while none out of 17 WT cells exhibited an EAD under the same experimental conditions.

Spontaneous APs and spontaneous SR Ca2+ release

During the same protocol, spontaneously occurring APs (sAPs; Fig. 6a) were recorded. These were defined as APs that were not triggered by an electrical current clamp command. sAPs were recorded in 8 out of 26 NCX-Tg myocytes with an average occurrence of 2.0 ± 0.6 sAPs per cell. No sAPs occurred in any of the 22 WT cells (Fig. 6c) during the same protocol. Characteristically, these spontaneous APs were initiated by a slow diastolic

depolarization as opposed to a sharp upstroke of the AP when triggered by a current clamp stimulus (see Fig. 6a inset).

The generation of spontaneous APs has been attributed to delayed afterdepolarizations (DAD) and these are thought to be triggered by spontaneous SR Ca^{2+} release events (sCR) that would then drive NCX inward current resulting in membrane potential instability and generation of a new, i.e., spontaneous AP [7, 9, 30, 52]. To investigate this hypothesis, Ca^{2+} transients were measured in a separate set of cells loaded with the Ca^{2+}-sensitive dye indo-1/AM that were field stimulated and subjected to the same pacing protocol as used above for voltage recordings. Per definition, Ca^{2+} release events that were not triggered by a field stimulus and showed at least one-third of the prior stimulated Ca^{2+}-transient amplitude were labeled as spontaneous Ca^{2+} release (sCR). Characteristic for sCR was a slow increase in diastolic Ca^{2+} concentration, which then developed into a full, spontaneous transient, whereas field-triggered transients had a sharp upstroke of the Ca^{2+} transient (Fig. 6b).

Interestingly, while sAPs exclusively occurred in NCX-Tg, sCR occurred in myocytes of both genotypes in a similar number per myocyte (NCX-Tg: 8.4 ± 2.0/cell; $n = 11$; WT: 7.4 ± 2.1/cell; $n = 5$; $p > 0.05$). The relative number of myocytes per genotype exhibiting sCR was smaller in WT (WT: 5 out 33; NCX-Tg: 11 out of 28; $p < 0.05$; Fig. 6c).

Discussion

The present study investigated the proarrhythmic potential as well as underlying proarrhythmic cellular and molecular mechanisms in a murine model with homozygous overexpression of the cardiac Na^+/Ca^{2+} exchanger. We here demonstrate that increased activity of NCX in a non-failing environment without reduced and even increased K_v prolongs AP duration, increases the likelihood of early and late afterdepolarizations in isolated cardiac myocytes and leads to an increased occurrence of ventricular tachyarrhythmias in the whole heart. These proarrhythmic effects are found prior to the development of morphological abnormalities or contractile dysfunction indicating heart failure.

Maintenance of Ca^{2+} homeostasis in NCX-Tg

Before discussing pathophysiological aspects of NCX overexpression, one has to define how Ca^{2+} homeostasis can be maintained in the face of increased Ca^{2+} extrusion. In our model, net Ca^{2+} influx is increased due to prolonged I_{Ca} activity. Thus, Ca_2^+ efflux and Ca^{2+} influx can be in balance. We suggest the following underlying mechanism by which increased I_{NCX} can influence I_{Ca}: Possibly, $_2^+$ concentration, increased I_{NCX} reduces dyadic cleft Ca resulting in reduced Ca^{2+}-dependent inactivation of I_{Ca}, resulting in prolonged I_{Ca} activity. This hypothesis is supported by previous studies by our group [31, 33].

Cardiac-specific overexpression of NCX causes proarrhythmia in the absence of cellular or whole heart failure

A previous study has characterized NCX-Tg mice at the age of 16 weeks and older. Using immunohistochemistry and telemetry, age-dependent propensity to cardiac failure under experimental conditions of physical stress was observed in postpartum females [38]. Another study has described alterations in contraction coupling at about the same age [37]. For the present study, all animals used were 12.5 weeks or younger and no postpartum females were used. No clinical signs of heart failure were observed. Echocardiography revealed unaltered cardiac function and heart weight/body weight ratio was also unaltered (Table 1). There was a non-significant trend toward increased left ventricular wall width. This may reflect a beginning development of cardiac hypertrophy as has been described for

this mouse model at higher age. We did not see any alterations in the expression levels of key Ca^{2+}-handling proteins characteristic for hypertrophy or heart failure when normalized to calsequestrin. Cell capacitance as a measure for cell size and thus a parameter for cellular hypertrophy was unaltered in NCX-Tg. Additionally, in NCX-Tg there was no reduction of the Ca^{2+} transient or increased diastolic Ca^{2+} as observed in most experimental models of heart failure. Hence, our findings were obtained in a situation devoid of heart failure.

Prolongation of cellular AP duration and arrhythmogenicity have been described to be associated with a reduction of K^+-carried outward currents and this mechanism has been suggested to be responsible for increased proarrhythmia in animal models of heart failure [30], in human failing myocardium [6] and in models of mutated K^+ channels in non-failing environments (for review see [17, 39]). In our model, the K_v is preserved and even increased (Fig. 4). Also, the deviation from normal repolarization occurs during the later phases of the AP (Fig. 3) where the maximum of NCX activity would be expected [8, 25]). Thus, since there is no decrease in K_v, the cellular AP prolongation and the cellular and whole heart proarrhythmic effect we see in NCX-Tg must be distinct from proarrhythmia involving a reduction of depolarizing K^+ currents as has been described for classical hypertrophy and heart failure proarrhythmic pathways.

To our knowledge, this is the first study to use a transgenic approach to investigate the proarrhythmic potential of NCX. We demonstrate for the first time that overexpression of NCX is an independent proarrhythmic factor that can cause EADs, spontaneous APs and ventricular tachyarrhythmia.

Molecular and cellular mechanisms underlying NCX-mediated proarrhythmia

The experiments presented in this study support the idea of more than one mechanism that leads from overexpression of NCX to proarrhythmia. Also, two different basic proarrhythmic cellular events, EADs and spontaneous APs, the latter triggered by (DADs), are observed in NCX-Tg and these will be discussed separately regarding their mechanism.

EADs: prolongation of the cellular AP—During a prolonged AP, the increased period of time in depolarized mode will broaden the "time window" for I_{Ca} or I_{Na} to become reactivated and thus trigger an EAD (for review see [17, 39]). In our model, EADs are exclusively observed in NCX-Tg. Even the experimental combination of an aggressive pacing protocol with catecholaminergic stimulation was not able to induce EADs in WT (Fig. 5). Thus—at least in our model and under the experimental conditions chosen—increased NCX activity seems to be an essential precondition for the development of EADs. The development of EADs does not require spontaneous—i.e., diastolic—SR Ca^{2+} release and thus the prolongation of the cellular AP due to increased NCX activity can be interpreted as an independent proarrhythmic mechanism that may have to be distinguished from the role of NCX in the generation of DADs as discussed below.

The observation that increased NCX activity would prolong the action potential is plausible since NCX generates an inward electrical current that would slow cellular repolarization. This has been supported experimentally by others [1, 26, 45] and by our group using transgenic murine models [31, 32]. NCX inward activity is directly dependent on its substrate Ca^{2+} and thus may be altered by altered systolic Ca^{2+}; however, in our model systolic Ca^{2+} is unaltered. The prolonged I_{Ca} activity as demonstrated in our model may add to the prolongation of the APD in NCX-Tg. Under certain conditions, NCX may work in reverse mode in which one Ca^{2+} ion enters the cell in exchange for three Na^+ ions (for review see [42]). However, it has been a matter of debate whether this outward current exerts a significant influence on the AP under near physiological conditions or only under pathophysiological or highly artificial experimental conditions. In our model, NCX reverse

mode does not seem to play a significant role since reverse mode NCX activity would generate an outward electric current and thus tend to reduce AP duration instead of prolonging it. The proarrhythmic mechanism suggested here—increased NCX activity resulting in a slowed cellular repolarization with QT prolongation and promotion of EADs—constitutes a novel experimental model of long QT syndrome. Our observations would attribute a similar proarrhythmic significance to NCX gain of function, as has been attributed to loss-of-function alterations of I_K in previously published long QT models. Dependence of AP duration on stimulation cycle length has been observed in several species [3]. In the isolated beating heart, there was a non-significant trend toward APD prolongation at lower frequencies in both genotypes, which was more pronounced in NCX-Tg. Hence, at lower heart rates, a rate-dependent prolongation of APD may contribute to the proarrhythmic effect in NCX-Tg.

Delayed afterdepolarizations: NCX inward current triggered by spontaneous SR Ca^{2+} release—NCX has also been attributed to be involved in the generation of DADs. Other than EADs which occur during the plateau or repolarization of the AP, DADs are temporary depolarizations that occur after full repolarization of the sacrolemma. The initial event leading to a DAD is believed to consist of a spontaneous release of Ca^{2+} from the SR. This increase in cytosolic Ca^{2+} would then lead to a sudden activation of NCX inward current, which would depolarize the sarcolemmal membrane to a potential from where a new and spontaneous AP is triggered. According to this hypothesis, the more NCX there is, the more easily would a given spontaneous Ca^{2+} release event translate into a DAD. Indeed, under baseline conditions, we observe spontaneous Ca^{2+} release events in both genotypes, whereas spontaneous APs are exclusively observed in NCX-Tg (Fig. 6c). This supports the hypothesis that the likelihood of spontaneous SR Ca^{2+} release to translate into DADs depends on the level of NCX expression. Our data show for the first time that an isolated upregulation of NCX—without reduced K^+ current or other changes observed in heart failure—is sufficient to increase the likelihood of DADs.

We have preliminary data showing that in the presence of isoprenaline, the number of sCR events increases in both genotypes and that DADs occur in both WT and NCX-Tg. Thus, as a limitation of the study, the effect we describe may not apply to conditions of catecholaminergic stimulation. Further studies will have to clarify this issue.

Increased occurrence of spontaneous SR Ca^{2+} release events in NCX-Tg—As discussed above, the translation of spontaneous SR Ca^{2+} release into DADs appears to be facilitated in NCX-Tg, which offers an explanation for the increased occurrence of DADs and spontaneous APs. However, although spontaneous SR Ca^{2+} release is observed in both genotypes, in NCX-Tg the number of affected cells is higher than in WT (Fig. 6c). A higher number of spontaneous Ca^{2+} release events will of course as well increase the likelihood of DADs just for the simple reason that there is more substrate for the development of DADs. This may be interpreted as a further independent mechanism of proarrhythmia. The mechanisms that come into question may either be increased SR Ca^{2+} load or increased RyR open probability. As shown in this study, SR Ca^{2+} load is unaltered in our model.

Thus, the possibility of increased RyR open probability as a consequence of increased NCX activity has to be considered. The main triggers for RyR-mediated Ca^{2+} release are Ca^{2+} ions. Although systolic and diastolic Ca^{2+} are unaltered in our model, measurements of global cellular Ca^{2+} may not reflect subtle temporary alterations of Ca^{2+} concentration in the dyadic cleft, where the Ca^{2+} binding site of RyR is located. Thus, enhanced RyR release activity resulting in increased sCR could be a direct consequence of altered dyadic cleft Ca^{2+} dynamics due to increased NCX and I_{Ca} activity as observed in our model.

Also, we detected an increased protein expression of RyR in our model (Fig. 1), which could provide an alternative explanation for proneness to sCR. The mechanisms underlying the regulation of RyR protein expression are largely unknown and there are contradicting observations as to whether RyR is increased, decreased or unaltered in cardiac pathology [10]. Increased RyR phosphorylation—as observed in our model—may as well contribute to enhanced sCR; however, it is controversial whether phosphorylation of Ser2808 can indeed increase RyR open probability [4].

Summary

The present work is the first to investigate the role of NCX in cardiac arrhythmia using a transgenic approach. Our results show that increased NCX activity results in a proarrhythmic phenotype before the onset of cellular or whole heart alterations characteristic for hypertrophy or heart failure. Increased NCX activity appears to be an independent proarrhythmic factor that slows cellular repolarization, increases the rate of EADs and DADs and causes ventricular tachycardias in the whole heart. In causing EADs and whole heart arrhythmia, gain of function of NCX in a murine model seems to have a similar proarrhythmic potential as loss of function of K_V, as observed in models of heart failure, hypertrophy or mutant models of LQT.

We suggest three distinct molecular mechanisms underlying NCX mediated proarrhythmia: (1) occurrence of EADs due to delayed repolarization; (2) an increased rate of DADs and sAPs caused by a facilitated translation of spontaneous SR Ca^{2+} release into DADs due to increased NCX activity; (3) a higher per se rate of spontaneous SR Ca^{2+} release possibly as a consequence of altered subsarcolemmal Ca^{2+} handling in NCX-Tg as reported previously.

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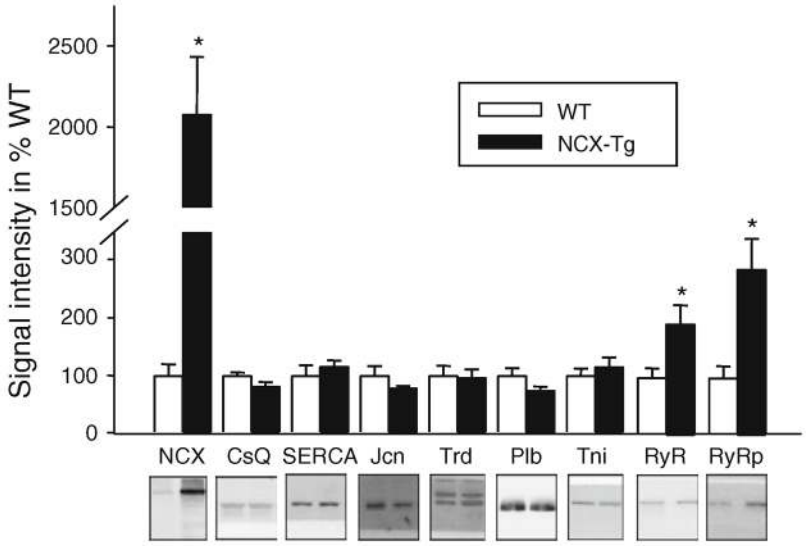

Fig. 1.

Protein expression in NCX-Tg versus WT (from *left* to *right*). Canine NCX (WT: $n = 5$; NCX-Tg: $n = 7$), calsequestrin (CsQ; here and for all following proteins WT: $n = 6$; NCX-Tg: $n = 6$), SERCA, *Jcn* junctin, *Trd* triadine, *Plb* phospholamban, *Tni* troponin I, *RyR* ryanodine receptor and *RyRp* phosphorylated RyR as assessed by Western immunoblot analysis and quantified by densitometry (*upper panel*). *Bottom* representative original immunoblot tracings. *$p < 0.05$ for WT versus NCX-Tg

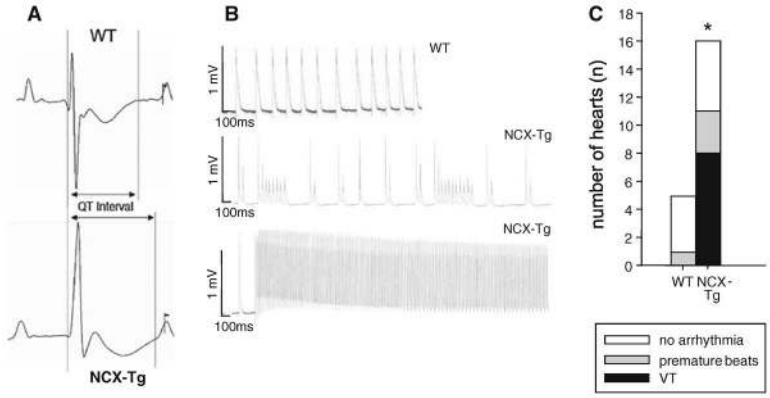

Fig. 2.

a Surface ECG from WT (*top*) and NCX-Tg (*bottom*) mice. Tracings demonstrated were generated by signal averaging from 20 ECG tracings per animal. **b** and **c** Proarrhythmic events in NCX-Tg compared with WT in isolated intact beating heart Langendorff preparations; **b** exemplary monophasic action potential tracings from WT and NCX-Tg with no arrhythmic events observed (*upper panel*), with premature beats and nonsustained VT (*middle panel*) and monomorphic sustained VT (*lower panel*). **c** Number of spontaneous arrhythmic events in NCX-Tg and WT mice. *$p < 0.05$ for WT versus NCX-Tg

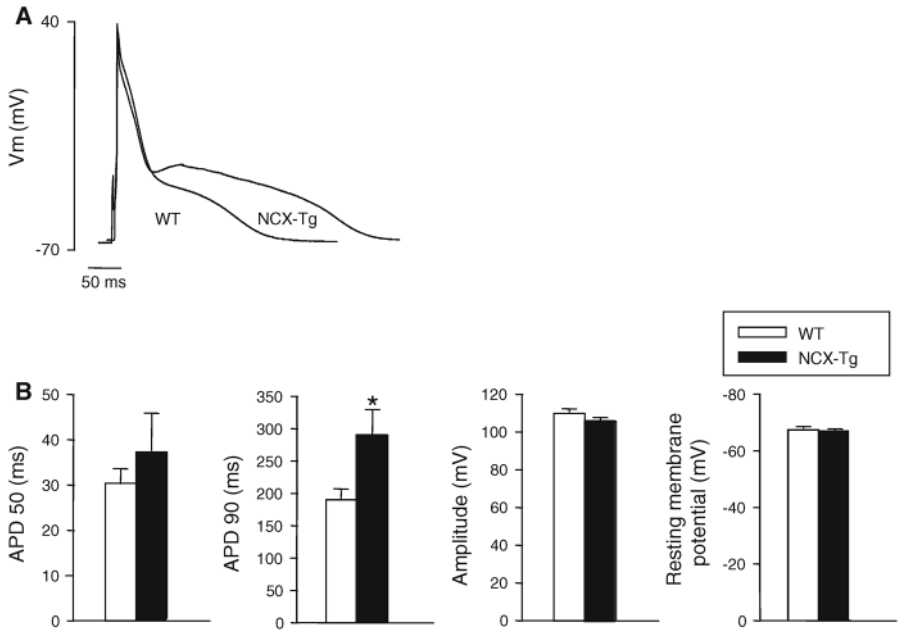

Fig. 3.

Prolonged cellular action potential duration recorded with the patch clamp method in current clamp mode in isolated NCX-Tg myocytes. **a** Representative cellular action potential recordings from NCX-Tg and WT myocytes. **b** Summary data on WT ($n = 22$ cells (15 animals)) and NCX-Tg ($n = 26$ cells (16 animals)) myocytes. *$p < 0.05$ for WT versus NCX-Tg

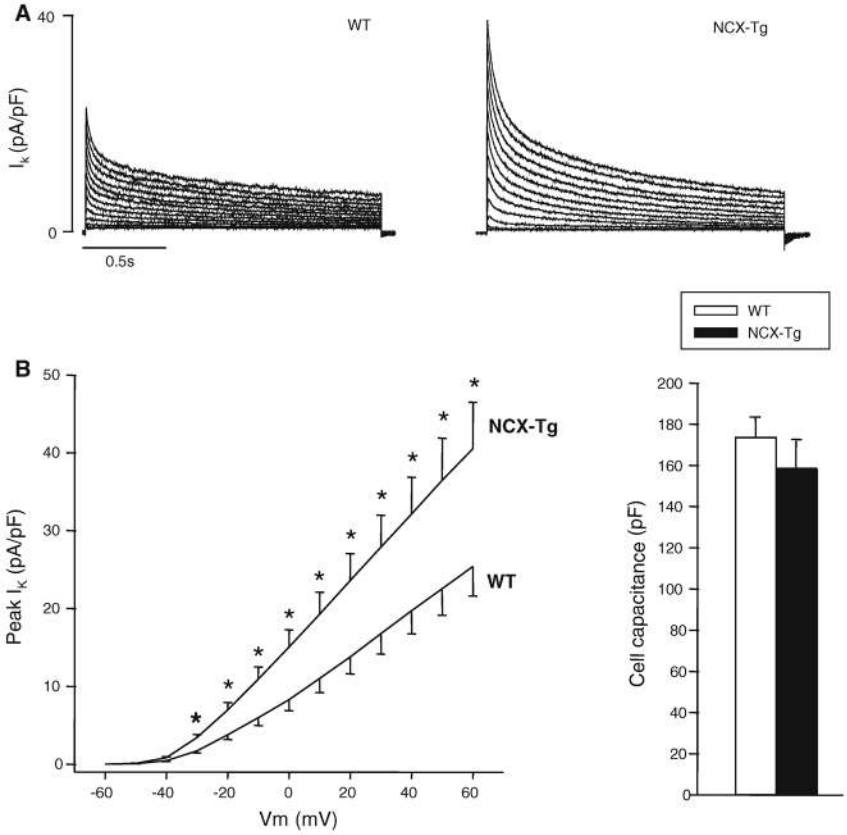

Fig. 4.

Increased activity of voltage activated transient K^+ current (I_K) in NCX-Tg versus WT. **a** Representative tracings normalized to cell capacitance from WT and NCX-Tg cardiac myocytes. Recordings were obtained with the patch clamp method in voltage clamp mode during test potentials ranging from −60 to +60 mV and a holding potential of −80 mV. **b** Quantification—*left* current–voltage relation of NCX-Tg ($n = 20$ cells (7 animals)) versus WT ($n = 19$ cells (10 animals)); *right* cell capacitance (*$p < 0.05$)

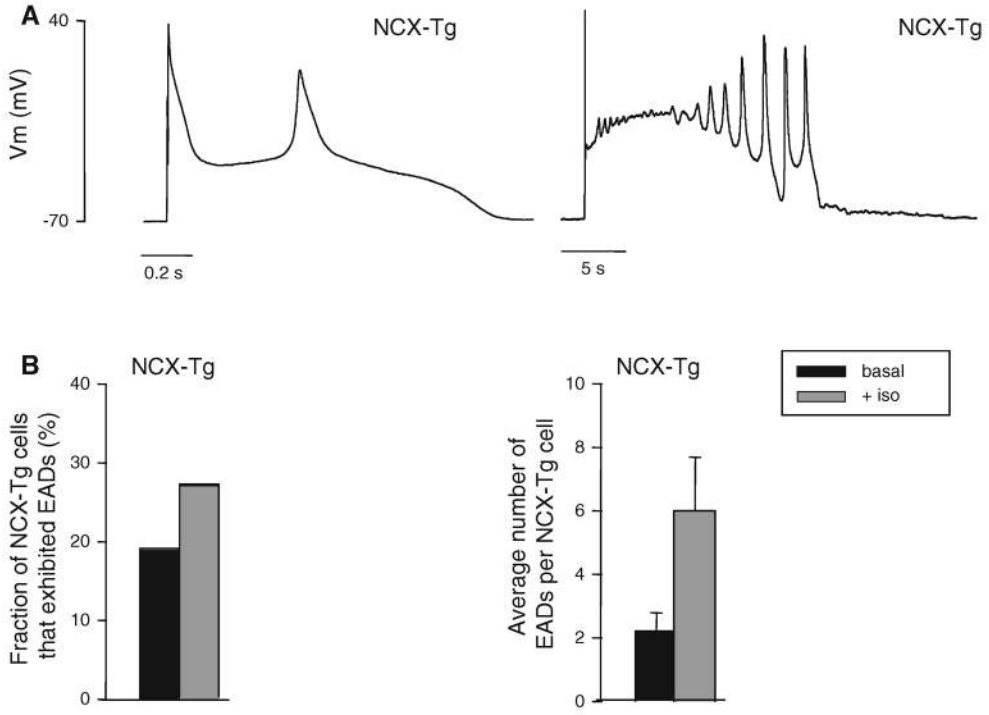

Fig. 5.

Early afterdepolarizations (EAD) are observed in NCX-Tg but not WT myocytes. Cells were paced in the current clamp mode with sudden changes in cycle length to provoke membrane instability [NCX-Tg: $n = 26$ cells (16 animals); WT $n = 22$ cells (15 animals)]. The experiments were repeated in the presence of isoprenaline (iso; NCX-Tg: $n = 22$ cells (14 animals); WT $n = 17$ cells (13 animals)). **a** Two examples of EADs recorded in isolated NCX-Tg myocytes. **b** Summary data on the occurrence of EADs in NCX-Tg in the absence and presence of isoprenaline. *Left* percentage of NCX-Tg myocytes exhibiting one or more EADs. *Right* average number of EADs occurring per cell in those NCX-Tg myocytes that exhibited one or more EADs

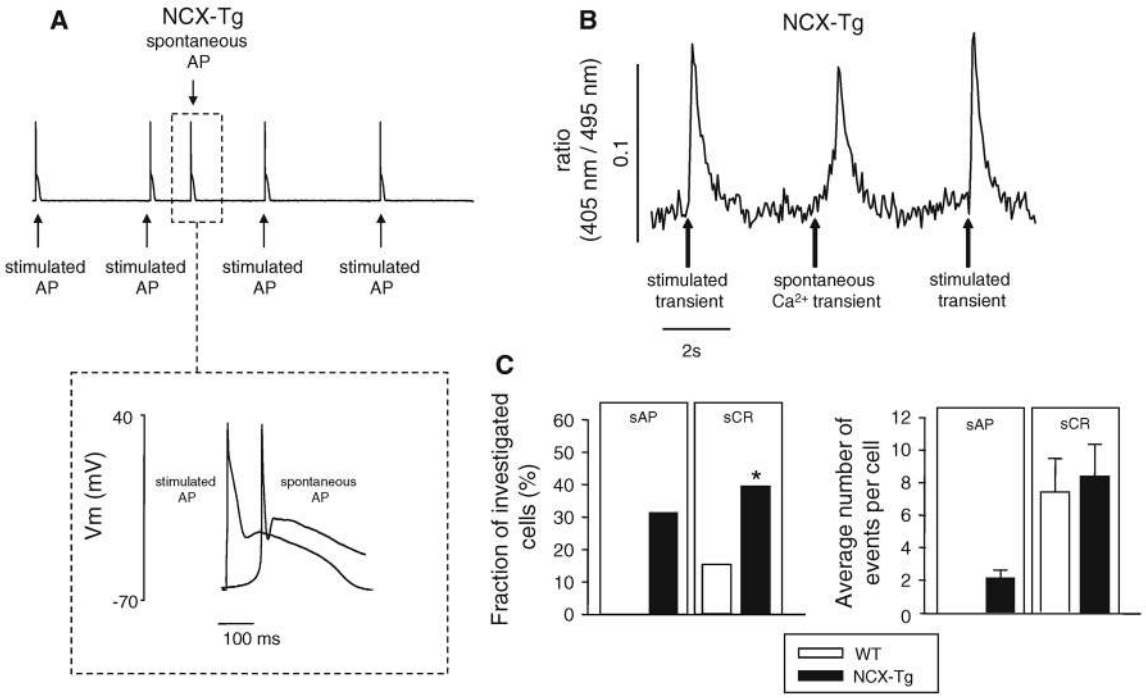

Fig. 6.

Occurrence of spontaneous APs (sAP) and spontaneous Ca^{2+} release (sCR). Membrane potential (NCX-Tg: $n = 26$ cells (16 animals); WT $n = 22$ cells (15 animals)) and Ca^{2+} transients (NCX-Tg: $n = 28$ cells (11 animals); WT $n = 33$ cells (17 animals)) were recorded during rapid changes of pacing cycle lengths. **a** An exemplary sAP recorded in a patch clamped NCX-Tg myocyte. Note the slow initiating diastolic repolarization as opposed to a sharp AP upstroke in APs triggered by a current clamp command (*inset*). **b** Example of sCR recorded in a field-stimulated NCX-Tg myocyte loaded with the Ca^{2+}-sensitive dye indo-1 AM. As opposed to Ca^{2+} release triggered by a field stimulus, sCR is initiated by a slow diastolic rise in cytosolic Ca^{2+}. **c** Summary data on the occurrence of sAPs and sCR. *Left* fraction of myocytes exhibiting one or more sAP or sCR, respectively. *Right*: Average number of sAP or sCR in those cells exhibiting sAP or sCR. While sAPs were observed exclusively in NCX-Tg, sCR was observed in myocytes of both genotypes. $*p < 0.05$ for WT versus NCX-Tg by χ^2 test

Table 1

Surface ECG and echocardiographic data from NCX-Tg and wild-type mice less than 12 weeks old

	WT mean	WT SEM	NCX-Tg mean	NCX-Tg SEM	$p < 0.05$*
Surface ECG					
HRs used for interval measurements (bpm)	602	8	582	8	
PQ (ms)	39	1	40	2	
Pdur (ms)	15	1	16	1	
QT (ms)	47	1	52	1	*
Echocardiography					
Fractional shortening (%)	33	3	26	2	
LV/BW ratio mg/g	4.2	0.2	4.2	0.2	
LV/Tibia ratio mg/mm	5.5	0.2	5.8	0.3	

* $p < 0.05$ for WT versus NCX-Tg. WT: $n = 6$; NCX-Tg: $n = 8$