Material and Methods

Canine Pacing-induced Heart Failure Model

Animal care and handling followed protocols approved by the Animal Care and Use Committee of the Johns Hopkins University. Dogs undergoing rapid pacing were sacrificed at the terminal stage of heart failure\(^\text{1,2}\). Procedures for induction of heart failure and isolation of ventricular myocytes were performed as described previously.\(^\text{1-3}\) Myocytes were isolated from the left ventricle by perfusion via left anterior descending or left circumflex coronary arteries. The myocytes carefully dissected from subendocardial and subepicardial layers (both less than 1-2 mm thickness) of left ventricle were referred to endomyocardial and epimyocardial myocytes, respectively.

Whole Cell Patch Clamp

The current was measured in ventricular myocytes using the whole-cell configuration of the patch clamp. Heat-polished patch pipettes fabricated from borosilicate glass capillaries (1.5 mm outer diameter, World Instruments) had a resistance of 1-3 M\(\Omega\) when filled with internal solution. All recordings were made at least 10-15 minutes after rupturing the membrane to allow for adequate internal dialysis. Voltage clamp was performed at 37 °C utilizing an Axopatch 200A patch clamp amplifier (Axon Instruments, Union City, CA). A holding potential of -40 mV was employed. A descending ramp voltage clamp protocol (dV/dt = 0.5 V/s) over a voltage range of 100 mV to -90 mV with a 10 second repetition interval was used. A liquid-junction potential
of \(-14.8\) mV was corrected. Currents were digitized at a rate of up to 2.5 kHz and low pass-filtered at 1 kHz. The external solution contained (in mmol/L): 140 NaCl, 1 MgCl\(_2\), 2 CaCl\(_2\), 5 HEPES, and 10 glucose, pH 7.40 with NaOH. Niflumic Acid (100 µmol/L), strophanthidin (10 µmol/L), nitredipine (10 µmol/L) were used to block Ca\(^{2+}\)-activated Cl\(^-\) channels, Na\(^+\)-K\(^+\) ATPase and L-type Ca\(^{2+}\) channels, respectively. The K\(^+\)-free internal solutions contained (in mmol/L) 20 NaCl, 19 CsCl, 110 cesium glutamate, 0.5 MgCl\(_2\), 5 Mg ATP, 20 HEPES, pH 7.25 with CsOH. The Ni\(^+\)-sensitive current (10 mmol/L NiCl\(_2\)) was taken as NCX current.

To validate the measurement of NCX current, lithium-based, zero Na\(^+\) and Ca\(^{2+}\) external solutions were employed. The control bath solution contained (in mmol/L): 125 LiCl, 0.5 EGTA, 1 MgCl\(_2\), 10 HEPES, 20 TEA, and 10 Glucose, pH 7.40 with LiOH. A different combination of LiCl (84.5 mmol/L), NaCl (40 mmol/L) and CaCl\(_2\) (0.5 mmol/L) were employed to activate the NCX current. The same concentrations of niflumic acid, strophanthidin and nitredipine as above were used. The internal solution contained (in mmol/L): 20 NaCl, 110 CsCl, 0.4 MgCl\(_2\), 5 Mg ATP, 10 HEPES, 20 TEA, 5 BAPTA, 1.75 CaCl\(_2\) (free \([Ca^{2+}]\), 0.133 µmol/L), pH 7.25 with CsOH. QX-314 (1 mmol/L) was included in the pipette solution to block Na\(^+\) currents. A holding potential of 0 mV was employed. An ascending ramp voltage clamp protocol (dV/dt = 0.5 V/s) over a voltage range of -100 mV to 60 mV with a 2 second repetition interval was used. A liquid-junction potential of -5 mV was corrected.
Real-Time Quantitative RT-PCR

Total RNA was isolated from canine ventricle using Qiagen RNAeasy. Fluorescence-based real-time reverse transcriptase-polymerase chain reaction (RT-PCR) was performed using a Perkin-Elmer Applied Biosystems Model 7900 sequence-detection system. The primers and Taqman probe (Applied Biosystems, ABI) were designed from the coding region exon 11 of NCX1 using Primer Express Software (ABI) (forward primer: 5’-TCCTCATGATTGGGCATACTGACA-3’; reverse primer: 5’-TTTCAAGCCGATGGTGCA T-3’; Taqman probe: 6-FAM 5’-CATTGGAGACCTGGCTTCCACTTTG-3’-TAMRA). Twenty nanograms DNase-treated total RNA from the test samples was amplified by Taqman One-Step RT-PCR reagents (ABI) and quantitatively analyzed by real-time PCR. The reaction mixture contained a total volume of 25 µl including each primer pair (0.2 µM for forward primer and 0.5 µM for reverse primer) and a Taqman probe (0.5 µM). The RNA samples were heated at 60°C for 3 min before being added to the reaction mixtures. The thermal cycling consisted of 48°C for 38 min for reverse transcription, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. An endogenous control gene 18S rRNA was used to normalize the quantification of the mRNA target for differences in the amount of total RNA added to each reaction. Two negative controls were employed including total RNA subjected to non-RT PCR, which is the reaction without reverse transcriptase, to rule out genomic DNA contamination and a sterile water sample subjected to RT-PCR in every experiment. Each reporter signal is divided by the fluorescence of an internal reference dye (ROX) to normalize for non-PCR-related fluorescence fluctuations.
**Western Blotting**

Proteins were extracted by homogenization as previously described\(^2\). NCX proteins were fractionated on NuPAGE Novex 7% Tris-Acetate Gel and transferred electrophoretically onto a nitrocellulose membrane (Invitrogen). Nonspecific antibody binding was blocked for 2 hours in 5% milk-TTBS (TBS containing 0.1% Tween 20). After washing, nitrocellulose sheets were incubated with the monoclonal anti-NCX1 antibodies (R3F1, 1:1000, Swant, Bellinzona, Switzerland) for 1 h at room temperature. Secondary antibody was sheep anti-mouse horseradish peroxidase conjugate (Amersham Pharmacia, UK). Protein bands were developed with ECL chemiluminescence (Amersham Pharmacia, Piscataway, NJ). Visualization and quantification of the bands were accomplished with the ImageQuant software (Molecular Dynamics). Calsequestrin (Research Diagnostics Inc, Flanders, NJ) was employed as control for normalization.

**Data Analysis**

Electrophysiological recording was acquired with customized software (Ionview, B. O’Rourke). Analysis was performed with customized software and Origin 6.0 (Microcal, Northhampton, MA). Unpaired Student’s \( t \) test and ANOVA were employed for the statistical analysis. \( P \) values less than 0.05 were considered statistically significant.
Results

To confirm whether the Ni\(^{2+}\)-sensitive currents represent the NCX current, a complimentary method with a lithium-based, zero Na\(^+\) and Ca\(^{2+}\) external solution was employed. Figure 1 illustrates the current (Trace A) recorded prior to activation of NCX in the absence of external Na\(^+\) and Ca\(^{2+}\) in a normal MID myocyte. Moreover, the control bath solution was Ca\(^{2+}\)-buffered. The background currents were similar to those observed previously\(^4\) and presumably are nonspecific background cationic currents with similar conductance for Na\(^+\) and Li\(^+\).\(^5\) By switching to the perfusate containing 40 mmol/L Na\(^+\) and 0.5 mmol/L Ca\(^{2+}\), NCX was quickly activated and the first full-developed NCX current was recorded (Trace B). Ten millimolar Ni\(^{2+}\) was applied to block the NCX current and the residual current (Trace C) was obtained. The current in the presence of Ni\(^{2+}\) (Trace C) nearly completely overlapped the current in the absence of Na\(^+\) and Ca\(^{2+}\) (Trace A), suggesting that the Ni\(^{2+}\)-sensitive current elicited by a ramp voltage-clamp protocol represents the NCX current.

Legend

**Online Figure 1. Control experiment of Ni\(^{2+}\)-sensitive current.** Trace A (black) was recorded at the beginning of experiment in the absence of external sodium and calcium. Trace B (blue) was acquired in the presence of 40 mmol/L Na\(^+\) and 0.5 mmol/L Ca\(^{2+}\). Ni\(^{2+}\) (10 mmol/L) was then applied and Trace C (red) was obtained at the end of the experiment.
References


A: 0 Ca\textsuperscript{2+}, 0 Na\textsuperscript{+}, Li\textsuperscript{2+}-substituted (Start)
B: 40 mM Na\textsuperscript{+}, 0.5 mM Ca\textsuperscript{2+}
C: 10 mM Ni\textsuperscript{2+}, 40 mM Na\textsuperscript{+}, 0.5 mM Ca\textsuperscript{2+} (End)