Supplementary Materials

Methods.

Cell Isolation. C57BL10 mice (wild-type, WT) and dystrophin-deficient *mdx* (C57BL/10ScSn-*mdx*) mice at the age of 1 (young), 3-4 (adults) and above 12-15 months old (senescent) were used in this study. Animals were purchased from the Jackson Laboratory. Ventricular myocytes from mouse heart were isolated as previously described[1]. Briefly, hearts were excised, cannulated and retrogradely perfused with (in mM): 140 NaCl, 5.4 KCl, 1.1 $MgCl_2$, 1 Na_2HPO_4, 5 HEPES and 10 glucose, pH was adjusted to 7.3 with NaOH at 37^0C. Cells were enzymatically dissociated with collagenase type II (Worthington) and protease (Sigma) treatment and used within 4-5 hours. All experiments were performed at room temperature and conformed with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (1996) and were approved by the Institutional Animal Care and Use Committee of the New Jersey Medical School of the University of Medicine and Dentistry of New Jersey and by the State Veterinary Office of Bern, Switzerland.

Intracellular Ca^{2+} responses to hypo-osmotic shock. Intact cardiomyocytes were loaded with fluo-3 AM (5 μM, Biotium) for 30 min at room temperature followed by at least 10 min of de-esterification. Cytosolic Ca^{2+} transients were recorded with fluo-3 and a laser scanning confocal microscope (Bio-Rad, Radiance 2000) in *XY*-scan mode at at the rate of 1 frame per 2 seconds. Fluo-3 was excited with the 488 nm line of an Argon laser and emission above 500 nm was collected. As a rule, 100 images were acquired: 10 images were taken in isotonic solution contained (in mM): 140 NaCl, 4 KCl, 1.8 $CaCl_2$, 1.1 $MgCl_2$, 10 glucose, 5 HEPES (further referred to as NT). Osmolarity was 305 mOsm and pH was adjusted to 7.4 with NaOH. Then 20 images were recorded in hypoosmotic solutions which contained 70 NaCl (instead of 140 NaCl) and consequently reduced osmolarity of 170 mOsm and the remaining 70 frames were recorded back in isotonic solution. The averaged intracellular Ca^{2+} responses during 60 sec after the osmotic shock were compared in different series of experiments.

Excitation-contraction coupling (electrophysiological recordings combined with confocal imaging). Membrane currents were measured using the whole cell patch-clamp procedures with an Axopatch 200B amplifier (Axon Instruments) controlled by custom-written data-acquisition software developed under LabView (National Instruments). Cells were voltage clamped using low-resistance (1.5–3 MΩ) borosilicate glass micropipettes pulled with a Zeitz DMZ puller (Zeitz Instruments, Germany) and filled with pipette solution which contained (in mM): 120 CsAsp, 8 NaCl, 20 tetraethylammonium (TEA)-Cl, 5.5 $MgCl_2$, 4 KATP, 5 HEPES, and 0.1 K_5-fluo-3. pH was adjusted to 7.2 with CsOH and osmolarity was 305 mOsm. Cells were held at -80 mV. After eight preconditioning pulses to 0 mV in order to equalize the SR Ca^{2+} load, a 400 ms test pulse to -25 mV was applied after an initial step to -40 mV to inactivate Na^+ and T-type Ca^{2+} currents. The SR loading protocol was applied in NT (with 1.8 $CaCl_2$) with a rapid switch to NT (with 0.5 $CaCl_2$) before the last preconditioning pulse. Simultaneously with Ca^{2+} currents intracellular Ca^{2+} transients were recorded in line-scan mode at a rate 750 lines/sec. The E-C coupling gain was calculated from the ratio of peak Ca^{2+} transients and peak Ca^{2+} currents at several tested clamp potentials and compared at different external Ca^{2+} concentrations

Estimation of SR Ca^{2+} leak. SR Ca^{2+} leak was estimated as described in[2]. Cells were paced at 1 Hz in external control solution to obtain steady-state levels of SR Ca^{2+} load, and starting from the last five beats, $[Ca^{2+}]_i$ was recorded in the line-scan mode (500 lines/s). After pacing, external solution was rapidly switched to Na^+ and Ca^{2+}-free solution (by substitution of Na^+ with Li^+ and addition of 0.5 mM EGTA) for 15 s to stop Ca^{2+} influx and to inhibit Ca^{2+} removal via the NCX and thus, to avoid Ca^{2+} overload of the cells. Addition of tetracaine (1 mM, 15 s) eliminated RyR-mediated diastolic Ca^{2+} leak, leading to a rapid decrease in $[Ca^{2+}]_i$ proportionally to the leak. The peak of the caffeine-induced Ca^{2+} transient measured after tetracaine washout (10 mM caffeine, 2 s) was used to estimate SR load.

Measurements of cytosolic ROS production and RyR free thiol content. For the quantification of ROS production, cells were loaded with 20 μM of 5-(and-6)-chloromethyl-20 , 70-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA, Invitrogen). CM-H_2DCFDA is hydrolysed to DCFH in the cell, and DCFH is oxidized to form highly fluorescent DCF in the presence of the appropriate oxidant (e.g. hydrogen peroxide or peroxynitrite). ROS/RNS generation was detected as a result of DCFH oxidation. As a rule, a series of 160 images was acquired at 0.5 Hz. CM-H2DCFDA was excited at 488 nm of Argon laser and emission was collected above 500 nm. Laser power was minimized to reduce the photoproduction of ROS. At the end of each experiment 10 mM H_2O_2 was added to determine maximum DCF fluorescence. The content of free thiols in RyR was determined using monobromobimane (mBB) fluorescence assay as described in[3]. Isolated cardiomyocytes were permeabilized, incubated with 1 mM of mBB for 1 hour in dark room and washed out 3 times to remove unbound mBB. The proteins were resolved in the 4-15% TGX SDS-gels (Bio-rad, CA) and transilluminated with UV light (excitation wavelength 360nm) by using MultiImage™ light cabinet (Alpha innotech, CA). Total RyR2 was determined from Coomassie blue staining of the gels run in parallel and confirmed by western blotting with anti-RYR2 (Thermo Scientific, MA).The location of the RyR band on this gel was also reassured with Mass Spectrometry at UMDNJ core facility.

Phosphorylation and Western Blots of RyR.
Mouse ventricular tissue was collected and sonicated in the radio-immunoprecipitation assay (RIPA) lysis buffer (1X TBS, 1% P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.004% sodium azide supplemented with phenylmethylsulfonyl fluoride PMSF, protease inhibitor cocktail and Calyculin A (Santa Cruz, CA)). Protein concentration in each sample was determined using an Eppendorf photometer with bovine serum albumin (BSA) as a standard. For immunoblotting, 40 µg of protein lysate per sample was denatured in 4X SDS-PAGE sample loading buffer. Proteins were separated by electrophoresis in to 4% (for RyR 2) and 10% (for CaMKII) respectively SDS-polyacrylamide gel, transferred to nitrocellulose membrane (Bio-Rad, CA) at 15V for 16-18 h at 4^0C (for RyR2) and at 90V for 15 h at 4^0C (for CaMKII), blocked with 1% BSA in TRIS buffered saline and Tween (TBS: 20mM TRIS-HCl, 200mM NaCl, 0.6% tween 20, pH 7.5) for 1 hour and probed for proteins of interest. Levels of RyR2, phosphorylated-RyR2, CaMKII and oxidized-CaMKII were assessed using specific primary antibodies: anti-RyR2 (1:500, Thermo Scientific, USA), anti-phosphorylated-RyR2-2808 (1:2000, Badrilla, UK), anti-phosphorylated-RyR2-2814 (1:500, Badrilla, UK), anti-CaMKII and anti-oxidized-CaMKII (1:500, both are a gift from Dr. M. Anderson) and appropriate HRP-conjugated secondary antibodies. Membranes were washed 3 times for 15 min in TBST after primary antibodies and secondary antibodies incubation respectively.The chemiluminescence was detected and acquired on to MultiImage™ light cabinet (Alpha Innotech, USA). GAPDH (1:1000, Millipore, USA) was probed as a protein loading control.

Data analysis and statistics. Images were pre-processed using the free software NIH ImageJ and further analyzed, together with electrophysiological data, in IgorPro Software (Wavemetrics). Results are shown as mean ± standard error of mean (SEM). All data sets contain results from a minimum of three mice (n on the bars indicates the number of cells). Statistical significance was evaluated by Student's t-test. A P-value of <0.05 was considered significant. On the graphs the significance is indicated by *.

1. Wolska BM, Solaro RJ. Method for isolation of adult mouse cardiac myocytes for studies of contraction and microfluorimetry. *Am J Physiol* 1996;**271**:H1250–1255.

2. Shannon TR, Ginsburg KS, Bers DM. Quantitative assessment of the SR Ca^{2+} leak-load relationship. *Circ Res* 2002;**91**:594–600.

3. Terentyev D, Gyorke I, Belevych AE, Terentyeva R, Sridhar A, Nishijima Y *et al.* Redox modification of ryanodine receptors contributes to sarcoplasmic reticulum Ca^{2+} leak in chronic heart failure. *Circ Res* 2008;**103**:1466–1472.

Table I. Pharmacology of Ca^{2+} signaling in *mdx* cardiomyocytes.

Ca^{2+} responses to osmotic shock, F/F_0				
Inhibitor	**Young (control)**	**Young (+inhibitor)**	**Adult (control)**	**Adult (+inhibitor)**
MPG	1.67 ± 0.13 (21)	1.36 ± 0.10 (17)	1.93 ± 0.18 (10)	1.33 ± 0.22 (9)
Apocynin	2.01 ± 0.18 (18)	1.50 ± 0.11 (22)	1.66 ± 0.13 (12)	1.28 ± 0.13 (13)
DPI	1.74 ± 0.12 (20)	1.21 ± 0.08 (13)	2.01 ± 0.15 (18)	1.43 ± 0.16 (9)
AIP	2.03 ± 0.15 (24)	1.38 ± 0.09 (24)	2.02 ± 0.15 (11)	1.30 ± 0.11 (11)
KN-93	1.65 ± 0.22 (13)	1.11 ± 0.04 (10)		
PKI	1.76 ± 0.09 (27)	1.56 ± 0.13 (24)	1.79 ± 0.17 (21)	1.16 ± 0.08 (13)
H89	1.56 ± 0.23 (16)	1.10 ± 0.15 (24)		
KT-5720	1.60 ± 0.31 (11)	1.44 ± 0.05 (10)		
SMLT	1.76 ± 0.16 (15)	1.69 ± 0.13 (19)	2.10 ± 0.19 (14)	1.48 ± 0.15 (14)
1400W	1.92 ± 0.14 (24)	1.70 ± 0.12 (27)	2.08 ± 0.15 (17)	1.45 ± 0.15 (13)
EC-coupling gain @-25 mV @0.5 Ca^{2+}, $(\Delta F/F_0)/(A/F)$				
Inhibitor	**Young (control)**	**Young (+inhibitor)**	**Adult (control)**	**Adult (+Inhibitor)**
MPG	0.81 ± 0.09 (8)	0.48 ± 0.05 (8)	1.38 ± 0.27 (8)	1.39 ± 0.24 (7)
AIP		0.63 ± 0.08 (6)		0.68 ± 0.07 (6)
PKI		0.87 ± 0.11 (5)		0.73 ± 0.17 (7)
SMLT		1.02 ± 0.22 (6)		0.93 ± 0.14 (6)
1400W		0.97 ± 0.22 (6)		0.99 ± 0.16 (5)
PTIO#				0.75 ± 0.11 (8)
SR Ca^{2+} Leak, %				
Inhibitor	**Senescent (control)**	**Senescent (+Inhibitor)**		
MPG	7.18 ± 0.54 (17)	5.66 ± 0.25 (8)		
AIP		4.99 ± 0.39 (8)		
PKI		5.49 ± 0.72 (7)		
SMLT		4.89 ± 0.35 (9)		
1400W		5.09 ± 0.32 (11)		

#NO scavenger PTIO (2-Phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide, 0.1 mM) was used to confirm results with NOS inhibitors.

Table II. Gradual deterioration of Ca^{2+} signaling in *mdx* cardiomyocytes.

Young		Adult		Senescent	
WT	***mdx***	**WT**	***mdx***	**WT**	***mdx***
Ca^{2+} responses to osmotic shock, F/F_0					
1.07 ± 0.01 (n=24)	1.74 ± 0.16 (n=18)	1.12 ± 0.02 (n=8)	1.88 ± 0.15 (n=18)	1.20 ± 0.01 (n=17)	1.80 ± 0.20 (n=16)
EC-coupling gain @-25 mV @0.5 Ca^{2+}, $(\Delta F/F_0)/(A/F)$					
0.45 ± 0.05 (n=5)	0.73 ± 0.05 (n=7)	0.60 ± 0.04 (n=7)	1.35 ± 0.27 (n=9)	0.55 ± 0.03 (n=6)	0.75 ± 0.08 (n=10)
SR Ca^{2+} Leak, %					
4.61 ± 0.25 (n=24)	5.50 ± 0.32 (n=18)	4.63 ± 0.27 (n=8)	6.56 ± 0.45 (n=18)	4.25 ± 0.26 (n=17)	7.18 ± 0.54 (n=16)
SR Ca^{2+} Load, F/F_0					
3.64 ± 0.16 (n=24)	4.32 ± 0.21 (n=18)	3.91 ± 0.39 (n=8)	3.32 ± 0.15 (n=18)	4.87 ± 0.21 (n=17)	3.06 ± 0.10 (n=16)