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SHORT COMMUNICATION

Testosterone Modulates Cardiomyocyte Ca^{2+} Handling and Contractile Function

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Summary

The extent to which sex differences in cardiac function may be attributed to the direct myocardial influence of testosterone is unclear. In this study the effects of gonadal testosterone withdrawal (GDX) and replacement (GDX+T) in rats, on cardiomyocyte shortening and intracellular Ca^{2+} handling was investigated (0.5 Hz, 25 °C). At all extracellular [Ca^{2+}] tested (0.5-2.0 mM), the Ca^{2+} transient amplitude was significantly reduced (by ~ 50 %) in myocytes of GDX rats two weeks post-gonadectomy. The time course of Ca^{2+} transient decay was significantly prolonged in GDX myocytes (tau, 455±80 ms) compared with intact (279±23 ms) and GDX+T (277±19 ms). Maximum shortening of GDX myocytes was markedly reduced (by more than 60 %) and relaxation significantly delayed (by more than 35 %) compared with intact and GDX+T groups. Thus testosterone replacement completely reversed the cardiomyocyte hypocontractility induced by gonadectomy. These results provide direct evidence for a role of testosterone in regulating functional Ca^{2+} handling and contractility in the heart.

Key words

Testosterone • Cardiac myocyte • Gonadectomy • Intracellular calcium • Cardiac contractility • Isotonic shortening

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There is increasing recognition of the sex differences in cardiac function and disease (Czubryt *et al.* 2006). Although poorly understood at present, there is experimental evidence of fundamental sex difference in myocardial excitation-contraction coupling and cardiomyocyte Ca^{2+} handling processes (Curl *et al.* 2001). Differences in male and female myocardial contractile reserve appears to be associated with sex differences in peak sarcoplasmic reticulum (SR) Ca^{2+} load and/or release during dynamic stress (Petre *et al.* 2007). In addition, age-related changes in excitation-contraction coupling in murine cardiac myocytes are differentially modulated by sex, involving changes in both plasma membrane and SR Ca^{2+} fluxes (Grandy *et al.* 2006).

Evidence supporting a role for testosterone in determining sex-based differences in cardiac function was provided by Golden *et al*. (2003) who have shown that gonadectomy in male rats results in a significant reduction in the expression of genes encoding the cardiac L-type Ca^{2+} channel (α_{1c}-subunit) and the Na^{+}/Ca^{2+} exchanger. These post-gonadectomy molecular changes occurred in parallel with slowed cardiomyocyte contraction and relaxation kinetics. The goal of the present study was to directly link testosterone withdrawal and replacement with functional alterations in both cardiomyocyte calcium handling and contractility in the male rat heart.

Gonadectomy (GDX) or sham-GDX was performed in 3-month-old male Wistar rats under anesthesia induced with ketamine (45 mg/kg i.m.) and xylazine (0.7 mg/kg i.m.). For animals in the testosterone replacement group (GDX+T) a silastic tubing pellet

(3 cm, 1.98 mm id x 3.18 mm od; Dow Corning Corporation, Midland, MI, USA) containing testosterone powder (Sigma, St Louis, MO, USA) was implanted subcutaneously at the mid-scapular region at the time of gonad surgery. Following surgery, all animals were allowed free access to food and water. All experimental procedures complied with the guidelines of the National Health and Medical Research Council of Australia on the Care and Use of Animals in Research and had the approval of the Monash University Animal Ethics Committee.

Two weeks after surgery rats were killed by decapitation under deep chloroform anesthesia. Adequacy of the GDX surgery and testosterone replacement (GDX+T) were determined by assessing prostate weight and by blood testosterone assay. GDX resulted in a significant decrease in prostate weight/body weight when compared with sham operated animals (0.15±0.01 mg/g vs. 0.92±0.13 mg/g, $p<0.002$), and testosterone replacement restored prostate weight to above control values (1.49±0.07 mg/g, $p<0.05$ compared to sham). Serum testosterone concentrations were determined using a direct, double-antibody ^{125}I radioimmunoassay kit (ICN Biomedicals, Coata Mesa, USA) validated for use with rats and mice. Serum testosterone in GDX animals was not detectable, whereas intact and GDX+T levels were 1.61±0.48 ng/ml and 2.18 ±0.26 ng/ml, respectively.

Single ventricular myocytes were isolated enzymatically as previously described (Curl *et al.* 2001). Briefly, hearts were removed and perfused in Langendorff mode with HEPES-buffered physiological saline solution (PSS) with the following composition (mM): NaCl 130; KCl 4.8; KH_2PO_4 1.2; $MgSO_4$ 1.2; $CaCl_2$ 1.5; HEPES (N-[2-hydroxyethyl]piperazine-N'-[2-ethane-sulfonic acid]) 10; glucose 10; pH 7.4; 37 ^oC. Perfusate was changed to nominally Ca^{2+}-free HEPES-buffered PSS to arrest mechanical activity immediately prior to the addition of collagenase (Worthington; Type II, 120-150 IU/ml), $CaCl_2$ (50 μM) and 2,3-butanedione monoxime (BDM, 25 mM). After 20 min the left ventricle was dissected, placed in a conical flask and myocytes were dispersed by gentle agitation. Fractions containing viable cells were resuspended at room temperature in 2-3 ml of HEPES-buffered PSS containing 1 mM Ca^{2+}, 2.5 % bovine serum albumin and 25 mM BDM and stored at room temperature. Cells were used within 8 h of isolation.

Cells were loaded with Fura-2 by 10-min incubation with 5 μM of the acetoxymethyl ester form (Fura-2/AM) (Molecular Probes, Eugene, OR, USA) at room temperature. An aliquot of Fura-2 loaded myocytes was placed in an experimental chamber (volume 600 μl) and allowed to settle. The chamber was mounted on the stage of a Nikon Diaphot 300 inverted fluorescence microscope and superfused at 0.7-1.0 ml/min with solution maintained at 25 ^oC. Fluorescence was recorded using a Cairn Spectrophotometer system (Cairn Research Limited, Kent, UK) coupled to the microscope fitted with an adjustable diaphragm to restrict the recording field to a single cell. Alternating excitation light at 340 and 380 nm was provided by a 75 W xenon lamp and rotating filter wheel and emitted fluorescence (510 nm) was recorded by a photomultiplier tube. Following current to voltage conversion the photomultiplier output was digitized and stored for analysis. The ratio of the fluorescence at 340 nm excitation to that at 380 nm ($R_{340/380}$, 100 Hz) was used to calculate the intracellular free $[Ca^{2+}]$. At the conclusion of the experiment an internal calibration was performed on each cell as previously described (Curl *et al.* 2001). The K_d of Fura-2 for Ca^{2+} taken to be 224 nM.

Myocyte shortening was monitored with a video edge detection system (Crescent Electronics, Sandy, UT, USA). Markers were placed at cell end-boundaries and the trigger threshold adjusted to track the movement of the cell during shortening. The voltage signal, offset by the edge detector, was computer-interfaced and digitally synchronized with the fluorescence signal for display and analysis.

For each cell, $[Ca^{2+}]_i$ was measured during steady-state stimulation at 0.5 Hz with the superfusate containing varying concentrations of extracellular Ca^{2+} (0.5, 1.0, 1.5 and 2.0 mM) without BDM present. With each change in extracellular $[Ca^{2+}]$, cells were allowed to equilibrate to the new $[Ca^{2+}]$ for 15 min before recordings were made. Data are presented mean ± S.E.M., and were analyzed using one-way ANOVA (including repeated measures for experiments where extracellular Ca^{2+} was varied). Statistical analyses were performed using SPSS V.13.0 (SPSS Inc., Chicago, IL, USA).

Peak $[Ca^{2+}]_i$ and the amplitude of the Ca^{2+} transient were markedly reduced in cardiac myocytes of GDX rats compared to the intact and testosterone replaced GDX+T groups (Fig. 1A). Mean values for the steady-state amplitude of the Ca^{2+} transient in intact, GDX, and testosterone-replaced GDX+T cardiac myocytes stimulated at 0.5 Hz and exposed to varying $[Ca^{2+}]_o$ are shown in Figure 1B. The amplitude of the

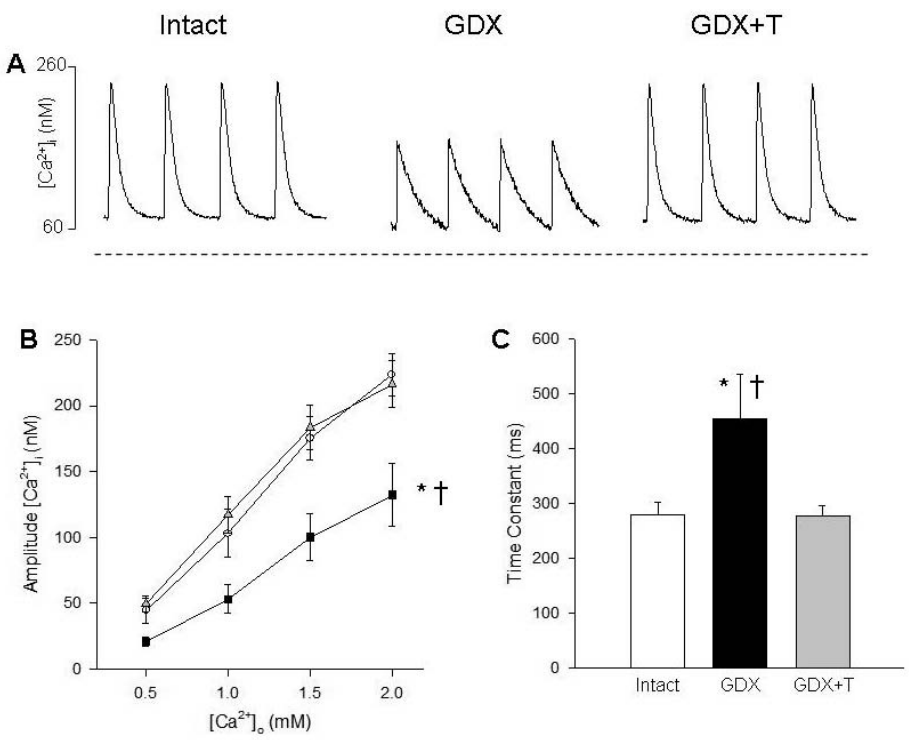

Fig. 1. A. Records of intracellular [Ca^{2+}] in single isolated cardiac myocytes of either intact, GDX, or testosterone-replaced GDX+T rats. Cells stimulated at 0.5 Hz in 1.5 mM Ca^{2+} PSS to contract at steady-state. **B.** The effects of increasing extracellular [Ca^{2+}] on the amplitude of the Ca^{2+} transient in intact (○), GDX (■) and testosterone-replaced GDX+T (▲) cardiac myocytes. Values are mean ± S.E.M. (12-15 cells/ group, 5-6 animals/group). **C.** Mean values for the time constant of decay of the Ca^{2+} transient in intact, GDX and testosterone-replaced GDX+T cardiac myocytes. Values are mean ± S.E.M. * $p<0.05$ vs intact, † $p<0.05$ vs GDX+T.

Ca^{2+} transient increased significantly ($p<0.001$) with elevated $[Ca^{2+}]_o$ similarly in all three groups. Across the range of extracellular Ca^{2+} concentrations, the amplitude of the Ca^{2+} transient was significantly lower (by ~ 50 %) in the GDX cardiac myocytes when compared with both intact ($p<0.003$) and testosterone-replaced GDX+T ($p<0.002$) cardiac myocytes. No significant difference between the intact and testosterone-replaced GDX+T cells was apparent. In addition to the reduced Ca^{2+} transient amplitude, the GDX male cardiac myocytes displayed significantly slower calcium transient decay (tau, 455±80 ms) when compared with both intact (279±23 ms; $p<0.05$) and testosterone-replaced GDX+T (277±19 ms; $p<0.03$) cardiac myocytes (Figs 1A and 1C). No significant difference between sham and testosterone-replaced GDX+T cardiac myocytes was apparent.

As presented in Figure 2, the effects of gonadectomy and testosterone replacement on the Ca^{2+} transient amplitude were paralleled by changes in cardiac myocyte shortening. Maximum cell shortening was markedly reduced in myocytes from the GDX group (2.7±0.6 %) when compared with both the intact (7.8± 1.8 %; $p<0.02$) and testosterone replaced GDX+T (7.2±1.3 %; $p<0.01$) groups (Fig. 2A and 2B). There was no significant difference in cell shortening between the sham and testosterone-replaced GDX groups. Time to 50 % cell re-lengthening in myocytes from the GDX group (235±35 ms) was significantly longer when compared with myocytes from the intact (133±15 ms; $p<0.02$) and testosterone-replaced GDX+T (146±15 ms; $p<0.03$) groups, while there was no detectable difference between the latter two groups (Fig. 2C).

This study provides the first demonstration that testosterone withdrawal *in vivo* profoundly and directly impacts myocardial excitation-contraction coupling by suppressing the cardiomyocyte systolic Ca^{2+} transient inducing a hypocontractile state. The markedly diminished amplitude and slower time-course of the Ca^{2+} transient in myocytes of gonadectomized animals coincided with similar reduction in peak cell shortening and relaxation delay. We show that *in vivo* testosterone replacement at physiologically relevant levels reverses these effects, characterizing testosterone as a major endogenous regulator of the cardiac inotropic state. We have previously reported sex differences in the Ca^{2+} transients of myocytes of non-gonadectomized rodents (Curl *et al.* 2001), and now we have identified that testosterone makes a major contribution to this sexually dimorphic phenotype.

The L-type Ca^{2+} channel may be a fundamental site through which sex-based differences in intracellular Ca^{2+} handling and contractility of cardiac muscle could arise. In male rats castration produces a 50 % reduction in myocyte dihydropyridine receptor mRNA levels which is prevented by testosterone treatment of castrated animals (Golden *et al.* 2003). Increases in L-type Ca^{2+} channel

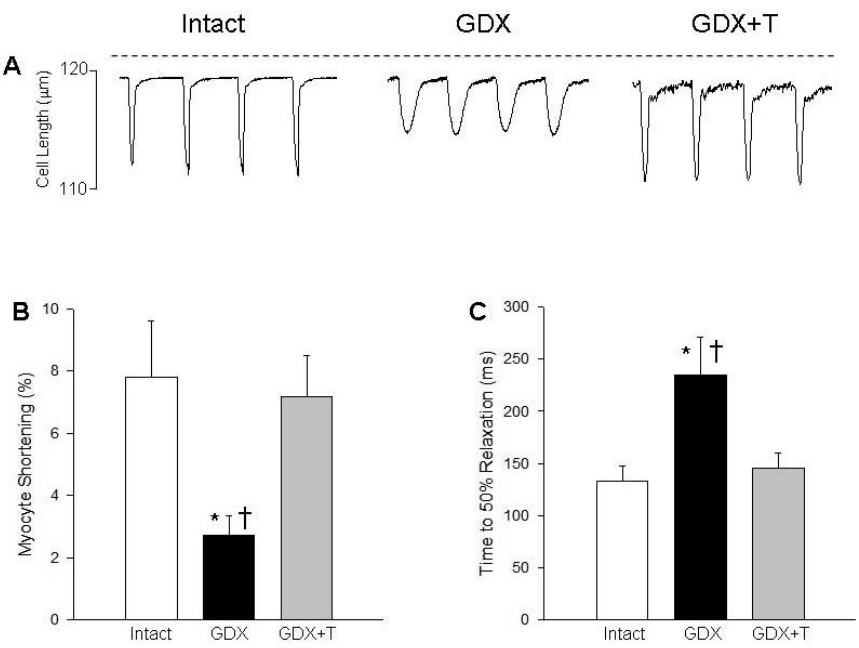

Fig. 2. A. Records of shortening in single isolated cardiac myocytes of either intact, GDX, or testosterone-replaced GDX+T rats. Cells stimulated at 0.5 Hz in 1.5 mM Ca^{2+} PSS to contract at steady-state. **B.** The extent of shortening in intact, GDX and testosterone-replaced GDX+T cardiac myocytes. Values are mean ± S.E.M. (12-15 cells/group, 5-6 animals/group). * $p<0.05$ vs intact, † $p<0.05$ vs GDX+T. **C.** Mean values for the time to 50 % cell re-lengthening in intact, GDX and testosterone-replaced GDX+T cardiac myocytes. Values are mean ± S.E.M. * $p<0.05$ vs intact, † $p<0.05$ vs GDX+T.

mRNA and contractility have also been reported in cultured rat cardiac myocytes exposed to testosterone for 8-24 h (Golden *et al.* 2004, Golden *et al.* 2005). Endogenous testosterone appears to be a primary regulator of the L-type Ca^{2+} channel in porcine coronary artery smooth muscle (Bowles *et al.* 2004). Thus, a reduction in L-type Ca^{2+} channel expression may underlie the decreased Ca^{2+} transient amplitude seen in cardiomyocytes of testosterone-deficient GDX male animals in this study.

The observed effects of gonadectomy and testosterone replacement on cardiac myocyte contractility, as detected by altered cell shortening in the present study, are consistent with previously reported findings (Golden *et al.* 2003). The decreased Ca^{2+} mobilization seen in the myocytes of gonadectomized animals in this study provides mechanistic explanation for the reduction in cell shortening. Relaxation was also slowed in the GDX male cardiac myocytes, as was the rate of decay of the Ca^{2+} transient. The slower rate of decay of the Ca^{2+} transient following gonadectomy suggests a decreased SR Ca^{2+}ATPase activity and/or Na^{+}/Ca^{2+} exchanger activity. A reduced SR Ca^{2+} load could also contribute to the differences in the Ca^{2+} transient apparent in the GDX myocytes. Whilst there currently appears to be no information available regarding possible effects of testosterone on SR Ca^{2+}ATPase, there is evidence for down-regulation of cardiac Na^{+}/Ca^{2+} exchanger gene expression by testosterone withdrawal in rats (Golden *et al.* 2003). Testosterone has also been shown to significantly upregulate Na^{+}/Ca^{2+} exchanger transcription in cultured neonatal cardiac myocytes (Golden *et al.* 2004). Sex differences in cardiomyocyte excitation-contraction coupling involving changes in both plasma membrane and SR Ca^{2+} fluxes have been reported (Grandy *et al.* 2006), and further investigation of the specific effects of testosterone are warranted. In particular, an influence of testosterone on β-adrenergic, PKA-induced phosphorylation of key Ca^{2+} transport proteins may be important in modulation of excitation-contraction coupling. Steroid regulation of myocyte Ca^{2+} flux mediated by sigma receptor activation and/or IP_3 signaling may also contribute to sex differences in cardiomyocyte contractility (Collier *et al.* 2007).

In summary, this study has identified a major influence of endogenous gonadal testosterone in modulating the cardiomyocyte Ca^{2+} transient and contractile response, providing direct evidence for a role of testosterone in regulating functional Ca^{2+} handling in the heart. Physiological levels of testosterone, as seen in the male, increase myocardial contractility by promoting intracellular Ca^{2+} mobilization. Thus testosterone directly contributes to sex-based differences in myocardial function and may also play a role in determining the differential incidence and outcomes of cardiac disease conditions in males and females.

Conflict of Interest

There is no conflict of interest.

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References

BOWLES DK, MADDALI KK, GANJAM VK, RUBIN LJ, THARP DL, TURK JR, HEAPS CL: Endogenous testosterone increases L-type Ca^{2+} channel expression in porcine coronary smooth muscle. *Am J Physiol* **287**: H2091-H2098, 2004.

COLLIER TL, WATERHOUSE RN, KASSIOU M: Imaging sigma receptors: applications in drug development. *Curr Pharm Des* **13**: 51-72, 2007.

CURL CL, WENDT IR, KOTSANAS G: Effects of gender on intracellular [Ca^{2+}] in rat cardiac myocytes. *Pflugers Arch* **441:** 709-716, 2001.

CZUBRYT MP, ESPIRA L, LAMOUREUX L, ABRENICA B: The role of sex in cardiac function and disease. *Can J Physiol Pharmacol* **84**: 93-109, 2006.

GOLDEN KL, MARSH JD, JIANG Y, BROWN T, MOULDEN J: Gonadectomy of adult male rats reduces contractility of isolated cardiac myocytes. *Am J Physiol* **285**: E449-E453, 2003.

GOLDEN KL, MARSH JD, JIANG Y: Testosterone regulates mRNA levels of calcium regulatory proteins in cardiac myocytes. *Horm Metab Res* **36**: 197-202, 2004.

GOLDEN KL, MARSH JD, JIANG Y, MOULDEN J: Acute actions of testosterone on contractile function of isolated rat ventricular myocytes. *Eur J Endocrinol* **152**: 479-83, 2005.

GRANDY SA, HOWLETT SE: Cardiac excitation-contraction coupling is altered in myocytes from aged male mice but not in cells from aged female mice. *Am J Physiol* **291**: H2362-H2370, 2006.

PETRE RE, QUAILE MP, ROSSMAN EI, RATCLIFFE SJ, BAILEY BA, HOUSER SR, MARGULIES KB: Sex-based differences in myocardial contractile reserve. *Am J Physiol* **292**: R810-R818, 2007.