Effects of Gonadectomy, Testosterone Replacement and Supplementation on Cardiac Action Potentials in the Rat

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Abstract

The aim of this study was to evaluate the electro-physiological alterations induced by gonadectomy, testosterone replacement and supplementation on cardiac action potentials (APs) in sexually mature male rats. Furthermore we studied the effects of testosterone deprivation and administration on total protein (TPC) and sialic acid content (SAC) of the left ventricle. Four groups of rats were compared: control (C), gonadectomized (GD), GD replaced with testosterone propionate (5 mg/kg i.m. 6 days/week for 4 weeks, GDTP) and testosterone supplemented rats (TP 5 mg/kg i.m. 6 days/week for 4 weeks). The APs recorded from GD rats both *in vitro* on right ventricle papillary muscles, at various stimulation rates, and *in situ* in subepicardial cells, at spontaneous heart rate, showed a significant lengthening of action potential duration (APD) measured at 10% (APD_{10}) and 50% (APD_{50}) of repolarization in comparison with TP, GDTP and C. A significant lengthening of the finale phase of relaxation (APD_{95}) was found also in TP rats. GD rats also showed a significant decrease in SAC. Testosterone replacement prevented both AP lengthening and SAC loss, whereas testosterone overload was associated only with a slight not significant increase of SAC. The rate at which ventricular fibrillation arises were determined and found to be lowered in both GD and TP groups when compared with C and GDTP rats. In conclusion, optimal testosterone serum level may contribute in the maintenance of normal myocardial electrogenesis and this may be partially attributed to regulation of the glycoprotein synthesis of glycocalyx.

Key words: action potential, androgens, glycocalyx, heart potential, sialic acid.

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Myocardial cells are targets for the action of testosterone and testosterone-like steroids. Androgen receptors have been identified in the myocardial cells of various animal species [18, 19]. Testosterone can modulate cardiomyocyte growth and myosin-ATPase [14, 24]. A wide range of effects mediated by androgens on cardiomyocyte membrane have been recently reported, including the stimulation of Ca fluxes and Ca dependent membrane transports [6, 11] and a direct impairment of the electrical coupling between cardiac muscle cells [28]. It has been recently shown that endogenous and exogenous androgens exert an important influence on the cell membrane function through a regulatory action on sialic acid containing glycoproteins and glycolipids of the outermost membrane leaflet [2, 9]. Sialic acid (N-acetylneuraminic acid), found on the external surface of cardiac myocytes [3, 31], binds strongly and preferentially ionised calcium at pH 7.3 and is essential for the preservation of electrical membrane properties [10, 22]. Neuraminidase induced removal of sialic acid from the surface of heart muscle cells, reduces the cell surface negative charge by 25% [26], modifies both T- and L-type Ca currents in senoatrial pacemakers cells and selectively modulates the function of the T- type Ca channel in ventricular myocytes [8, 33]. Moreover, depletion of sarcolemmal sialic acid produces a large increase in cardiac myocyte Ca uptake and cell contracture [16]. These data suggest that glycosilation of membrane macromolecules may influence membrane electrical properties and voltage dependent channel function possibly by influencing the local electric field detected by voltage sensors [4].

All these findings suggest that testosterone may influence the sarcolemmal electrical properties of ventricular myocytes possibly by controlling the glycoprotein synthesis of glycocalyx. The purpose of this investigation was to obtain more insights into the effects of long term castration and testosterone administration on the electrical activity of cardiac myocytes and to assess possible correlations between these effects and changes in sar-

colemmal sialoprotein. The interest of this study is enhanced by the need to elucidate whether electrophysiological alterations can explain unfavourable cardiac events, included ventricular fibrillation, associate with anabolic-androgenic steroid abuse [20].

Materials and Methods

Experimental groups

Male adult Wistar rats, 12 weeks old, were randomly assigned to one of the following four groups: control (C), gonadectomized (GD), GD replaced with testosterone propionate (5 mg/kg i.m. 6 days/week for 4 weeks, GDTP) and testosterone supplemented (TP; 5 mg/kg i.m. 6 days/week for 4 weeks) groups. The animals, born and maintained at the facilities of the Institute of Human Physiology of the University of Pavia, were kept in separate cages with pellet diet and free access to food and water during treatment. At the end of the experimental period (4 weeks), spontaneous heart rate was determined by means of electrocardiographic recordings under slight ether anaesthesia. The day of the experiment the animal was weighted and anaesthetized for action potential recording either *in situ* or *in vitro* as described below.

Testosterone serum level determinations

At the time of sacrifice the adequacy of gonadectomy was determined by documenting the seminal vesicles atrophy. Blood samples were withdrawn and were kept on ice 1h before centrifugation at a temperature of 4°C. Serum samples were collected and stored at –80°C until hormone assays were performed with commercially available radioimmunoassay kit (Testosterone CT, Diagnostic System Laboratories, Webster, Texas, USA).

In situ *recording of action potentials and determination of the fibrillation threshold*

At the end of the treatment period, the animals, fasted overnight, were anaesthetised with urethane (1 g/kg, i.p.). The heart was exposed through a left thoracotomy and pericardial sac dissection. Respiration was maintained by a rodent ventilator (7025, Basile Biological Research Apparatus, Comerio, Varese, Italy) connected to a tracheal cannula (ED 2 mm, ID 1.4 mm) with stroke volume of 2 ml at 70 strokes P.M. The ventricular myocardial cells were impaled using the floating technique previously described by Benoit (1962) [5] and Rindi et al. (1970) [23] i.e. with standard borosilicate microelectrodes (Clark Electromedical Instruments, Reading, UK) supported by a polythene flexible mount (Portex Limited, Hythe, Kent, UK), both filled with 3M KCl. All microelectrodes used had an electrical resistance of 20-30 MΩ and an apex less than 1 µm. The action potentials were amplified with an intracellular IE-201 electrometer (Warner Instrument, Hamden, USA), displayed on the screen of a Nicolet 4094C digital oscilloscope (Madison, Wisconsin, USA) and stored on floppy disks. Ventricular action potentials were recorded first at spontaneous heart rate and then at 400 and 500 beats/min obtained by application of electrode pulses (3 msec, 30 V) to the right atrium through a bipolar platinum electrode connected to a pulse stimulator with stimulus insulator and constant current. Impalament was maintained at each frequency for about 10 sec; the heart rate was allowed to revert to the spontaneous beating for about 1min before being raised to the next higher frequency. At the end of the recording period the stimulation rate was progressively increased from spontaneous heart rate until ventricular fibrillation begun. For each action potential the following parameters were analysed: amplitude (A), duration at 10% and 50% of repolarization (APD_{10} and APD_{50}) and maximal upstroke velocity (Vmax).

In vitro *recording of action potentials*

Rats under ether anaesthesia were sacrificed by decapitation and the hearts rapidly excised and placed in 95% O_2/5% CO_2 bubbled modified Krebs solution of the following composition: 120 mM NaCl, 2.4 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, $NaHCO_3$ 24.8 mM, glucose 1 g/l, pH 7.4.

The anterior right papillary muscles were then removed and placed in a recording chamber between two bipolar silver electrodes and continuously perfused at a rate of 5 ml/min with pre-warmed oxygenated Krebs solution (24°C).

Action potentials were recorded with conventional glass microelectrodes (tip resistance 10-15 MΩ) filled with 3M KCl. Transmembrane potentials were measured by means of an electrometer amplifier connected to an AT-mio-E10 board (National Instruments) for personal computer to record and store data. Signals were analysed using Labview 5.1 software (National Instruments). An equilibration time of 1h at a stimulation frequency of 0.1 Hz preceded the impalement. During each recording session the frequency of stimulation was increased to 0.2, 0.5, 1 and 2 Hz. The resting membrane potentials (Vm) and the following parameters of the action potentials were analysed: action potential amplitude (A), overshoot (OS), duration at 10% (APD_{10}), 50% (APD_{50}) and 95% (APD_{95}) of the repolarization and maximal upstroke velocity (Vmax).

Sialic acid and protein assay

At the end of the electrophysiological recordings, the left ventricles were cut, weighed and homogenized using a Potter S homogenizer (B.Braun, Melsungen AG, Germany). An acid hydrolysis of the homogenates was performed using the method described by Vatier et al. (1988) [27]. Total protein assay was carried out using the method of Lowry et al. (1951) [15] with bovine serum albumin as a standard. Protein content is expressed as mg of proteins per mg of wet ventricular weight. Si-

alic acid content was evaluated using a modification of the thiobarbituric acid assay previously described by Warren (1959) [29] and Aminoff (1961) [1]. This is a colorimetric assay in which sialic acids are oxidised with sodium periodate. The oxidation product is therefore coupled with thiobarbituric acid and the resulting chromophore extracted into cyclohexanone. Optical densities of the organic phase were determined at 549 nm using a Beckman spectrophotometer.

Results of sialic acid assay are expressed as µg of sialic acid per mg of tissue proteins.

Drugs and chemicals

Testosterone propionate, urethane and bovine serum albumin were purchased from Sigma. All the other reagents were purchased from BDH Chemicals.

Statistical analysis

Data were expressed as means ± S.E. Comparison between the experimental groups was performed using ANOVA followed by the Student-Newman-Keuls procedure. Significance was established at $P < 0.05$.

Results

Body and heart weight and testosterone serum level

Table 1 shows the values of initial and final body weight, body weight increase, heart weight, and testosterone serum levels evaluated at the time of sacrifice. All treated rats exhibited a significant decrease in body weight growth in comparison with controls: both hormonal alterations and the stress due to the everyday injection might have contributed to the reduced growth. Interestingly heart weight did not decrease and a clear hypertrophy was present in TP rats. As expected, gonadectomy was associated with a marked decrease in testosterone serum level, which was brought back to control values by the hormonal replacement. On the other hand, testosterone supplementation was coupled with a striking increase in testosterone serum concentration.

Protein and sialic acid content of the left ventricle

Figure 1 shows the average concentrations of sialic acid and of protein in the left ventricles of the four experimental groups. Gonadectomy caused a significant decrease in sialic acid content in comparison with C, TP and GDTP; the decrease was completely abolished by testosterone replacement. Testosterone supplementation was not able to increase further the sialic acid content. As sialic acid content was referred to protein content, it was of interest to see whether the possible changes in

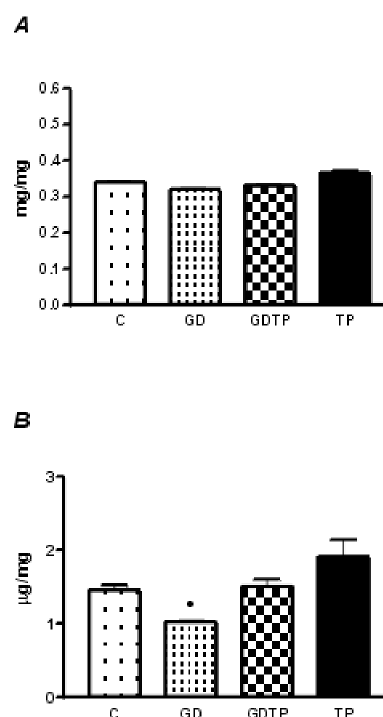

Figure 1. Effects of gonadectomy and testosterone propionate supplementation on protein (A) and sialic acid content (B) of rat left ventricular myocardium. Means ± S.E. C = Controls, GD = Gonadectomy, GDTP = Testosterone replacement to gonadectomized rats; TP = Testosterone supplementation. • = significantly different from C, GDTP and TP groups, P < 0.05.

Table 1. Body parameters and testosterone serum levels.

	C	GD	GDTP	TP
IBW (g)	383±9	372±12	361±10	399±27
FBW (g)	500±9	426±16	422±14	452±18
Δ BW (g)	104±10	59±6*	74±10*	61±8*
HW (g)	0.99±0.03	1.01±0.05	1.07±0.06	1.25±0.05**
HW/BW.100	0.198	0.237	0.252	0.275
TSL (ng/ml)	1.70±0.2	<0.1	2±0.6	23.8±6.0**

C = control; GD = gonadectomized; GDTP = gonadectomized replaced with testosterone propionate; TP = testosterone treated. IBW = initial body weight, FBW = final body weight, delta BW = body weight increase, HW = heart weight, TSL = testosterone serum levels. Means ± S.E.; n = 10 except for TLS where n = 5; * = significantly different from C; ** = significantly different from the other groups, $P < 0.05$.

protein anabolism in relation to lack or presence of testosterone might have modified the protein content of the cardiac tissue in the four experimental groups. The ventricular protein content appeared to be slightly decreased in gonadectomized rats and slightly increased after chronic testosterone overload, but in no case the differences were statistically significant.

In situ *recorded action potentials*

The parameters of the action potentials recorded in the four experimental groups at spontaneous and externally increased heart rate are showed in Figure 2. Upon externally increase of heart rate, a decrease of depolarization rate by about 30% in comparison with spontaneous heart rate occurred in all experimental groups. The time course of the early phase of repolarization (APD_{10} and APD_{50}) was significantly delayed in GD rats at spontaneous heart rate in comparison with C, GDTP and TP groups. This difference decreased when the heart rate was externally increased to 400 b/min and disappeared at 500 b/min. No differences in APD and Vmax emerged after testosterone overload.

Fibrillation threshold was determined increasing progressively the heart rate until fibrillation arose. Interestingly, the threshold for fibrillation was significantly lowered in GD as well as in TP rats in comparison with C and GDTP groups (C: 801.8 ± 4.8; GD: 706 ± 33.3; GDTP ± 790.7 10.4; TP: 665.7± 18.79 b/min n = 10, $P < 0.05$).

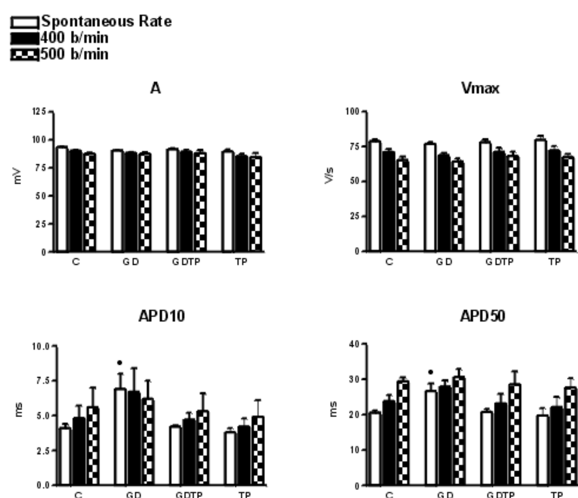

Figure 2. Action potential parameters (A, amplitude; Vmax, maximal upstroke velocity; APD10, action potential duration at 10% of repolarization; APD50, action potential duration at 50% of repolarization) recorded in situ at spontaneous and externally increased heart rate at 400 and 500 b/min. Means ± S.E. C = Controls, GD = Gonadectomy, GDTP = Testosterone replacement to gonadectomized rats; TP = Testosterone supplementation. • = significantly different from C, GDTP and TP groups, P < 0.05.

In vitro *recorded action potentials*

Data concerning membrane and action potential characteristics collected at 0.2 and 2 Hz are summarised in Figure 3. At all frequencies of stimulation gonadectomy and testosterone supplementation did not significantly affect resting potential, amplitude and overshoot of the action potentials whereas APD_{10} and APD_{50} significantly increased after gonadectomy in comparison with C, TP and GDTP rats. The delayed phase of repolarization (APD_{95}) was prolonged both in GD and TP preparations when compared to C and GDTP groups but only in TP group changes were statistically relevant. Figure 4 shows typical superimposed traces of intracellular action potentials recorded from controls, gonadectomized and testosterone treated rats. After castration the prolongation of the early phase of repolarization phase is clearly visible (left panel). Testosterone supplementation induced a prolongation of the delayed phase of repolarization (right panel).

Discussion

In this study we aimed to assess the effect of gonadectomy, testosterone replacement and testosterone sup-

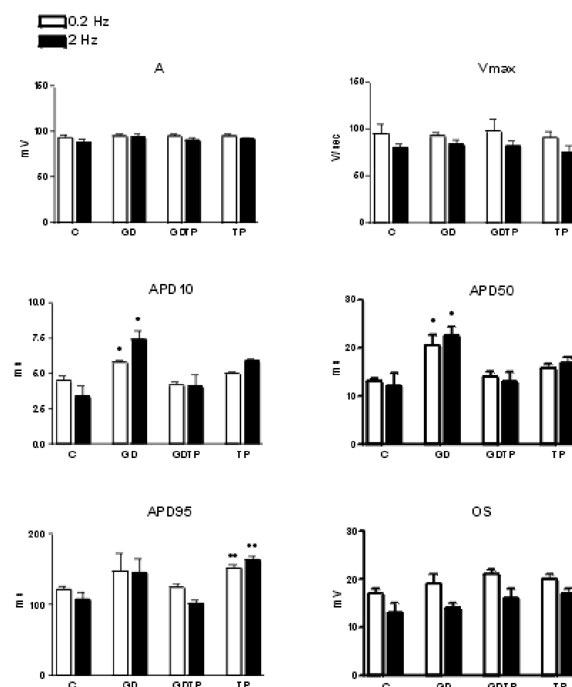

Figure 3. Action potential parameters (A, amplitude; Vmax, maximal upstroke velocity; APD10, APD50 and APD95, action potential duration at 10, 50 and 95% of repolarization; OS, overshoot) recorded in vitro at 0.2 and 2 Hz. . Means ± S.E. C = Controls, GD = Gonadectomy, GDTP = Testosterone replacement to gonadectomized rats; TP = Testosterone supplementation. • = significantly different from C, GDTP and TP groups; • • = significantly different from C and GDTP, P < 0.05.

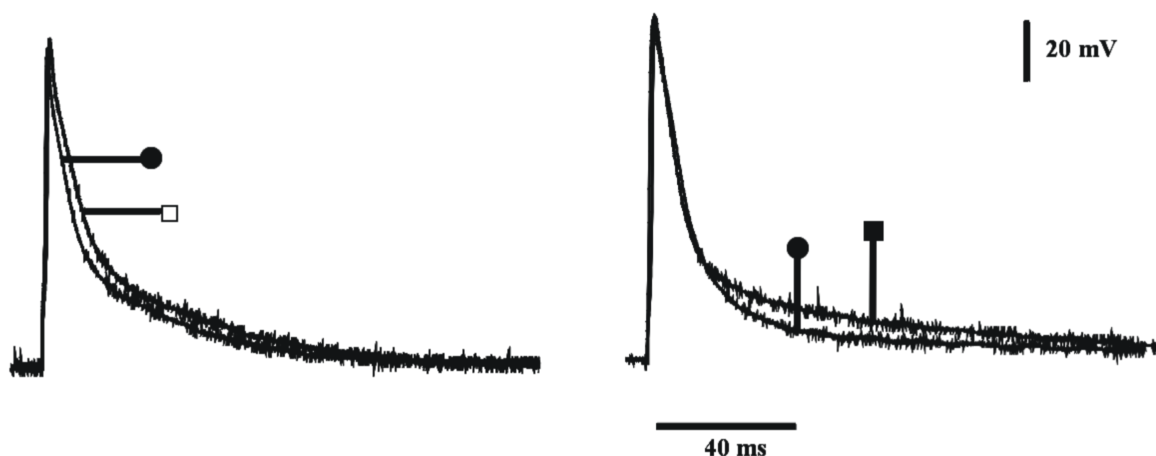

Figure 4. Superimposed intracellular action potentials recorded in vitro at 0.2 Hz. • = Controls, □ = Gonadectomized rats, ■ = Testosterone supplementation.

plementation on cardiac cell electrical activity and evaluate whether possible alterations were correlated with the sialic acid content of the tissue. The action potentials of ventricular cardiomyocytes were recorded *in situ* from subepicardial myocytes and *in vitro* from the anterior right papillary muscles of the rat heart at different frequencies of stimulation. Testosterone deprivation produced an APD lengthening in the early phase of the action potential (APD_{10} and APD_{50}) suggesting the presence of disorders of repolarization. In testosterone supplemented animals action potentials recorded *in vitro* also showed a prolongation of the delayed phase of repolarization (APD_{95}). The delays in repolarization were not associated with modifications of resting membrane potential and action potential amplitude. These findings suggest that the APD lengthening can be attributed to current changes instead of modifications of the activation state of channels involved in membrane repolarization. This is also supported by persistence of APD modifications of the *in vitro* recorded action potentials at various stimulation frequencies. Surprisingly in *in situ* conditions, externally increase of heart rate is associated with non-significant prolongation of APD_{10} and APD_{50}. This evidence may be due to a greater variability of APD, in castrated rats, at very high frequencies of stimulation or to a heterogeneity in the action potential modifications between subepicardial myocytes and right papillary muscles. In the rat, the early stage of repolarization is mainly due to long lasting Ca inward current and K outward currents as transient outward current (ITO) and inwardly rectifying potassium current (IK1), respectively involved in the early and late portions of the action potential plateau. The late phase of repolarization is mainly due to delayed rectifier potassium currents (iK). A possible explanation for APD changes in the early phase of repolarization observed in castrated rats resides in a loss of glycocalyx anionic charges, through a down regulation of sialic acid containing glycoprotein synthesis. As already mentioned, the oligosaccharides of the external cell coat play a crucial role in maintaining trans-membrane Ca fluxes and the negative fixed charges of glycocalyx act as a filter to Ca ions, thus regulating the cellular calcium uptake [32]. A loss in sialic acids may increase the long lasting Ca current promoting a significant delay in the early phase of repolarization. Gonadectomy produced a significant decrease in sialic acids assayed in left ventricles, which were restored after testosterone replacement. This suggests that the effects of testosterone deprivation on glycocalyx composition are, at least partially, due to a direct involvement of androgens in the regulation of sialic acid containing proteins. On the other hand testosterone overload failed to induce modifications of sialoprotein and total protein concentration. In agreement with recent observations that androgens, as testosterone and dihydrotestosterone, can induce a marked receptor-dependent hypertrophic response in rat myocytes [17], testosterone treated rats in the present study showed a significant increase of the heart weight and of heart weight/body weight ratio. In spite of the lack of changes of sialic acid ventricular content, the delayed phase of repolarization was prolonged in TP rats. This delay might be due to the known APD prolongation induced by cell hypertrophy, which is mainly ascribed to an increase of L-type Ca current in the early stage of hypertrophy and a decrease of voltage-gated K channels in the advanced stage of hypertrophy [13]. Moreover APD changes in overloaded rats may be also due to a direct involvement of testosterone in the development of downregulated potassium channel expression in ventricular cells as already shown in association with a prolongation of the QT interval in the rabbit heart [7].

A major finding of this investigation is that both castrated and testosterone overloaded rats exhibited an increased susceptibility to fibrillation. This can be related to the observed modifications of the action potential profiles. In presence of APDs lengthening, the time duration of the ventricular functional refractoriness may

compromise the wave front propagation along the heart tissue. This may impair the myocardial functional syncytium properties and let re-entrant excitations to arise. The change in wavelength (conduction velocity multiplied by the effective refractory period) of re-entrant circuits is known to play a role in the generation of functional re-entry, particularly in presence of sudden changes in rhythm such as when the pacing rate is rapidly increased. Furthermore, removal of sialic residues induces delayed after-depolarization and triggered activity through a greater calcium influx [10] and in testosterone overloaded rats the lowering of the input resistance induced by cellular hypertrophy may also contribute to wavelength changes of re-entrant circuits.

In summary, the results of this investigation demonstrate that testosterone deprivation exerts significant modifications of cardiac action potentials and a loss of sialic acids in ventricular myocytes. Both alterations are restored by testosterone replacement. On the other hand testosterone overload is not associated with modification of sialic acid content of the left ventricle, but still some alterations of the repolarization phase are present.

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