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Arrhythmogenic effect of androgens on the rat heart

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Abstract In most species androgens shorten the cardiac action potential and reduce the risk of afterdepolarizations. Despite the central role of the rat model in physiological studies, the effects of androgens on the rat heart are still inconclusive. We therefore performed electrophysiological studies on the perfused rat right ventricular free wall. We found a correlation between androgenic activity and a propensity to generate ventricular ectopic action potentials. We also found that the testosterone treatment increased action potential duration at 90 % of repolarization (APD90), while androgenic inhibition increased the time to peak and decreased APD90. We observed that the voltage-gated potassium channel Kv4.3 and the bi-directional membrane ion transporter NCX in the rat myocardium were regulated by androgenic hormones. One possible explanation for these findings is that due to the expression of specific ion channels in the rat myocardium, the action potential response to its hormonal background is different from those described in other experimental models. Our results indicate that androgenic control of NCX expression plays a key role in determining arrhythmogenicity in the rat heart.

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Introduction

Action potential duration (APD) is shortened by androgens in humans [1], Guinea pigs [2], and mice [3] among others. At least part of this action is caused by a reduction of the duration of repolarization, which in turn is determined by the activity of several potassium currents (I_{Kr}, I_{Ks} and I_{K1}) [4]. Higher activity of these currents shortens repolarization while lower activity increases it. Androgens increase the density of these currents at both the genomic [5] and non-genomic levels [6]. The effects of androgens on APD in the rat remain controversial: Leblanc et al. found no changes in the APD as a function of gender [7], while in a series of experiments done with androgenic steroids, Medei et al. found that they increased APD [8].

Androgens have also been found to reduce the risk of afterdepolarizations [9], an action which appears to hold true in most species [3, 6, 10] but which has not been properly confirmed in rats, a widely used model in electrophysiological studies. Kuhar et al. observed that in rats, testosterone (as well as estradiol) pretreatment prevented arrhythmia in a setting of ischemia–reperfusion in Langendorff preparations [11]. Additional studies point to an androgen-induced increase in arrhythmogenesis in the rat [8, 12]. We consider that interpretation of these findings and of others are strongly influenced by our understanding of the experimental models. As such, the rat model should be subjected to a more thorough study.

The AP in the rat is strikingly short and lacks a significant calcium ion (Ca^{2+}) plateau. This has led to repolarization in rat AP being frequently considered as being

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divided into a fast repolarizing phase immediately followed by a slow repolarizing phase (Fig. 2a). This sequence is the consequence of the specific set of ionic currents that predominate in the heart tissue of this species. Following phase 0, the main component of the fast repolarizing phase is a strong calcium-independent transient outward current (Ito). In rats, Ito is the result of the activity of a number of channels and regulators, including the voltage-gated potassium channels Kv4.2, Kv4.3, and, to a minor degree, Kv1.4 [13]. The intensity and kinetics of Ito are also subject to regulation by different factors. Expression of Kv4.3 is modulated under pathological conditions, such as heart failure [14]. Thyroid hormone studies indicate that the expression of Kv1.4, Kv4.2, and Kv4.3 in the heart are also hormonally regulated [15].

Another component of the fast repolarizing phase is the sodium ion $(Na^+)/Ca^{2+}$ transporter NCX, a bi-directional membrane ion transporter, operating in reverse mode. In most species during phase 1, and in some species during early phase 2, the equilibrium potential of NCX is above that of the membrane and, consequently, the exchanger is operating in the reverse mode, generating a repolarizing current [16]. As such, this current may contribute to the shape of the notch following the finalization of phase 0. Below a certain voltage (also depending on Ca^{++} and Na^+ concentrations), repolarization continues at slower rate. In rats, the key currents mentioned above for humans, rabbit, and mouse are absent (I_{Kr}, I_{Ks}) or at least smaller (I_{K1}). As a result, the slope of this final, slow repolarization is in large part the result of the activity of NCX [17]. In this phase, NCX is usually working in a forward mode to generate a depolarizing current; therefore, an increase in NCX activity should lengthen the AP.

In summary, as a consequence of its alternating modes (direct in phase 0, reverse in the fast repolarizing phase, and direct again in the slow repolarizing phase), the effect of NCX activity on the shape of the AP would be to shorten the fast repolarizing phase and to extend the slow repolarizing phase [18].

Rats are a commonly used model in cardiac physiology research, but the effects oft gender on rat APD or arrhytmogenicity is not clearly defined. The aim of this study was (1) to determine whether androgens have any effects on arrhythmogenicity in the rat heart and (2) to explain these effects, if any, based on changes in the AP caused by androgens.

Methods

Animals

Three-month-old male Wistar rats were used for the experiments. The animals were maintained under controlled temperature and humidity conditions on a 12:12-h light:dark cycle, with food and water ad libitum. The euthanasia procedure consisted of sedating the animal with inhalation anesthesia (5 % Inhelthran® enflurane; Abbott S.R.L., Rome, Italy), followed by cervical dislocation and isolation of the heart. The anesthesia was considered to be adequate when there was an absence of reflexes prior to cervical dislocation. This investigation was approved by the University of San Martin ethical review committee and conforms to the guidelines in the "Guide for the care and use of laboratory animals" [National Research Council, National Academies Press, Washington DC (1996)].

RNA isolation and real time PCR

Total RNA was isolated from the right ventricles of the isolated hearts with TRIzol (Invitrogen, Carlsbad, CA). Reverse transcription was carried out using random primers supplied by Biodynamics SRL (Buenos Aires, Argentina) and RevertAid M-MuLV Reverse Transcriptase (Fermentas, Thermo Fisher Scientific, Waltham, MA), according to the manufacturers' instructions. The real time PCR reaction was carried out using FastStart Universal SYBR Green Master (Rox) (Roche Life Science, Indianapolis, IN). The cycling program consisted of one cycle at 50 °C for 2 min, one cycle at 95 °C for 10 min (first denaturation), one cycle at 95 °C for 20 s (denaturation), one cycle at 60 °C for 1 min (annealing), and one cycle at 72 °C for 20 s (extension). Expression of mRNAs was normalized to the expression of glyceraldehyde 3-phosphate dehydrogenase mRNA. Data were analyzed using the Pfaffl method [19]. The primer list is given in the Electronic Supplementary Material.

Protein isolation and western blot

For protein isolation, one-half of the right ventricle was homogenized in 1 ml of RIPA buffer (150 mM NaCl, 10 mM Tris pH 7.2; 0.1 % sodium dodecyl sulfate, 1 % Triton X-100, 5 mM EDTA) and protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO), and the resultant homogenate was centrifuged at 15,000 g for 15 min at 4 °C. Samples were stored at -80 °C for later use. Protein concentration was determined using the dye-binding technique of Bradford. Western blot analysis was performed with anti-Kv4.3 (Alomone Labs, Jerusalem, Israel), anti-NCX (Santa Cruz Biotechnology, Dallas, TX), anti-β tubulin (loading control; Santa Cruz Biotechnology), and anti-rabbit immunoglobulin G secondary antibody conjugated to alkaline phosphatase (Santa Cruz Biotechnology). Membranes were analyzed using the Beta 4.0.3 Scion Image Software (Scion Corp., Frederick, MD). Kv4.3 and NCX abundance was calculated as the ratio of the density

of the western blot band divided by the density of the β-tubulin band. In all figures, relative abundance for each gene has been normalized to the mean ratio of the control group.

Drug administration

The animals were divided into three treatment groups for the molecular analysis to evaluate the role of androgenic hormones on Kv4.3 and NCX expression. Control animals were injected with vehicle only (200 μl corn oil, 1 % ethanol). The three treatment groups were: the finasteride (a 5-α-reductase inhibitor) group, with rats receiving an intramuscular injection of finasteride 2 mg (Sigma-Aldrich) [20]; the flutamide [an androgen receptor (AR) competitive inhibitor] group, with rats receiving an intraperitoneal (i.p.) injection of flutamide 10 mg (Sigma-Aldrich, USA) [21]; the testosterone group, with rats receiving an intramuscular injection of testosterone 5 mg (Sigma-Aldrich) [22]. All drug doses were adjusted for maximal transcriptional effect. The injections were administered once a day for 1 week.

Electrocardiography

Rats were anesthetized with sodium pentobarbital (50 mg/kg; i.p.). Electrocardiography leads were recorded with surface electrodes. Wave amplitudes were measured in millivolts, and the duration of each interval was measured in milliseconds. The mean value for each rat was obtained from 60 values consisting of 60 consecutive cardiac cycles using MATLAB R 2011B (MathWorks Inc., Natick, MA). QT intervals were measured from the beginning of the QRS complex to the end of the T wave.

Arterially perfused right ventricle

Experiments were performed on preparations of the isolated right ventricular wall. Following excision of the heart from euthanized animals, the heart was excised and the right ventricle isolated and retrogradely perfused through the aorta artery with Tyrode's solution at 37 °C. Perfusion was confirmed by the absence of areas retaining erythrocytes, as previously described [23]. Internal controls to determine the suitability of the preparation included the sustained response to pacing stimulus at 0.5, 1, and 5 Hz, and the presence of a stable resting membrane potential of <-60 mV. APs were recorded from the epicardial layer of the right ventricle using a floating microelectrode filled with 3 M KCl (FD223 electrometer; World Precision Instruments Inc., Sarasota, FL). Signals were digitized at a sampling rate of 1 kHz. All drugs were dissolved in Tyrode's solution and infused into the preparation via the aorta.

The preparation was stimulated using a square pulse of 3-ms duration at 5 Hz (Pulsemaster A300; World Precision Instruments Inc.). The arrhythmic pacing protocol (APP) was 10 Hz for 10 s, 20 Hz for 10 s, and 1 min at 0.5 Hz.

In the context of this model we defined the ventricular extrasystolic action potential (VEAP) as any AP that was not the result of direct stimulation. The APP was considered to be positive when it resulted in a VEAP.

Statistical analysis

Results are expressed as mean ± standard deviation of the mean. Data were analyzed by analysis of variance with Newman–Keuls a posteriori or Bonferroni correction as appropriate. The frequencies of positive APP were compared against the control group according to their binomial distribution. Differences were considered to be statistically significant at $p < 0.05$.

Results

The four groups displayed different androgenic activities, and the aim of our experiments was to evaluate androgenic control of transcriptional activity whenever possible. In terms of androgenic activity, the testosterone-supplemented rats showed the highest activity, followed in decreasing order of activity by the control rats, the rats treated with the 5-α-reductase inhibitor finasteride, and lastly the rats treated with the AR competitive inhibitor flutamide (which inhibits AR transcriptional activity but not non-genomic androgenic activity).

Androgenic hormone activity affects arrhythmogenicity in rat ventricular myocytes

Isolated right ventricular wall preparations were made from five rats from each group and the preparations treated with the 18-24 APP. The response to stimulation with this APP varied among groups (Fig. 1). In control rats, 30 % of the APP induced VEAP, while in testosterone-supplemented rates the VEAP frequency increased to 50 %. In comparison, the hearts from rats treated with finasteride and flutamide had lower frequencies of positive APP at 11 and 17 %, respectively (all differences are against control values and were significantly different by cumulative binomial probability, $p < 0.05$) (Fig. 1b).
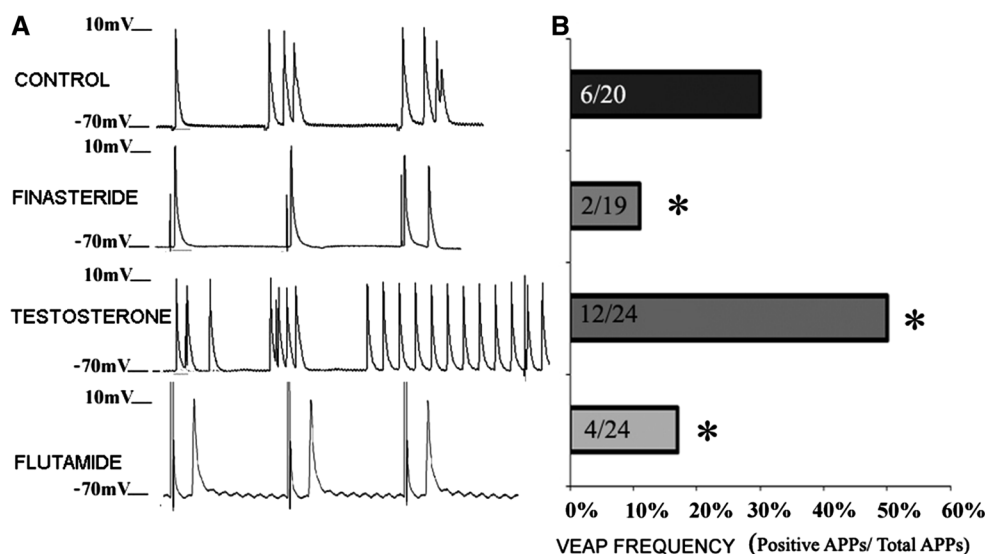
Fig. 1 Androgenic hormones activities regulate arrhythmogenicity. **a** Representative action potentials (AP) recorded after applying the arrhythmic pacing protocol (*APP*; 10 Hz for 10 s, 20 Hz for 10 s and 1 min at 0.5 Hz) to isolated and perfused right ventricles excised from rats of each group ($n = 5$ rats per group). **b** Bar graph showing the percentage of positive APP for each group. *Numbers inside bars* Number of positive APPs/total APPs. *Asterisk* Significantly different from control at $p < 0.05$ by cumulative binomial probabilities. *VEAP* Ventricular extrasystolic action potential. For a description of the groups, see "Drug administration"

Effect of androgenic hormone activity on the cardiac action potential

The AP from eight rats from each of the four groups with different androgenic hormone activities are shown in Fig. 2b. A certain correlation between androgenic hormone activity and time to peak (TTP) was observed. Testosterone supplementation did not change the TTP compared to the control (3.09 ± 0.11 vs. 2.9 ± 0.14, respectively), while the TTP for both the flutamide group (3.62 ± 0.11 ms) and finasteride group (3.6 ± 0.15 ms*) were significantly longer than that of the control ($p < 0.05$). This longer TTP in the flutamide and finasteride groups is likely the reason for the longer QT interval in these groups (QT: control 63.6 ± 3.4 ms; testosterone 62.6 ± 4.2; finasteride 75.0 ± 1.1; flutamide 76.5 ± 1.68). After reaching 50 % of repolarization (APD50), all differences were lost (APD50: 8.78 ± 0.4, 10.28 ± 0.76, 8.61 ± 0.28, and 10.27 ± 0.26 ms for the control, testosterone, flutamide, and finasteride groups, respectively). At 90 % of repolarization (APD90), the control and finasteride groups were not significantly different (APD90: 28.51 ± 2.15 vs. 30.57 ± 1.15 ms, respectively), while in the testosterone group the APD90 had increased by 55 % versus the control (44.32 ± 2.41 vs. 28.51 ± 2.15, respectively; $p < 0.05$) and in the flutamide group the APD90 was 25 % shorter versus the control (21.35 ± 0.72 vs. 28.51 ± 2.15; $p < 0.05$).

We further explored the kinetics of repolarization in the rat AP by modeling fast and slow repolarization times as a function of the percentage of repolarization from the maximum amplitude. Fast repolarization (from APD10 to APD50) displayed a linear behavior in all groups ($R^2 > 0.9$), as shown in Fig. 3a where the intercept corresponds to the TTP and the slope is inversely proportional to the velocity of repolarization (ms/%).

The slope of the fast repolarization phase for the control group was $11.82 \pm 0.89 \times 10^{-3}$ ms^{-1}. None of the slopes for the other groups differed significantly from that of the control (testosterone $13.69 \pm 1.64 \times 10^{-3}$ ms^{-1}; flutamide $9.128 \pm 1.12 \times 10^{-3}$ ms^{-1}; finasteride $12.74 \pm 0.63 \times 10^{-3}$ ms^{-1}) (Fig. 3a).

The slow repolarization phase (from APD50 to APD90) was adequately described by an exponential function of the form $F(x) = A_0 \times e^{kx}$. In this case, the exponential factor K represents the rate constant and is the slope of the semi-log graph (Fig. 3b). The rate constant for the function that describes the AP of the control group was $29 \pm 3.4 \times 10^{-3}$ ms^{-1}. Augmented androgenic activity in the testosterone group did not result in a significant increase in the exponent ($36.5 \pm 3.5 \times 10^{-3}$ ms^{-1}) of the adjusted function when compared with the control; nevertheless, we observe significant differences when comparing the testosterone to the flutamide group ($23.8 \pm 1.5 \times 10^{-3}$ ms^{-1}) or the finasteride group ($28.8 \pm 2.1 \times 10^{-3}$ ms^{-1}) ($p < 0.05$).

## Control of Kv4.3 and NCX by androgenic hormones

To study the control of Kv4.3 and NCX by androgenic hormones we examined five male Wistar rats from each of

Fig. 2 Androgenic hormones regulate the duration of the AP. **a** Representative AP from all four groups ($n = 8$ rats per group). **b** Effect of androgenic hormone activity on AP variables (*TTP* time to peak, *APD50* AP duration at 50 % of repolarization, *APD90* AP duration at 90 % of repolarization, *QT* duration of QT interval measured from the beginning of the QRS complex to the end of the T wave). *Asterisk* Significantly different from control at $p < 0.05$. **c** Representative electrocardiography results for the four groups
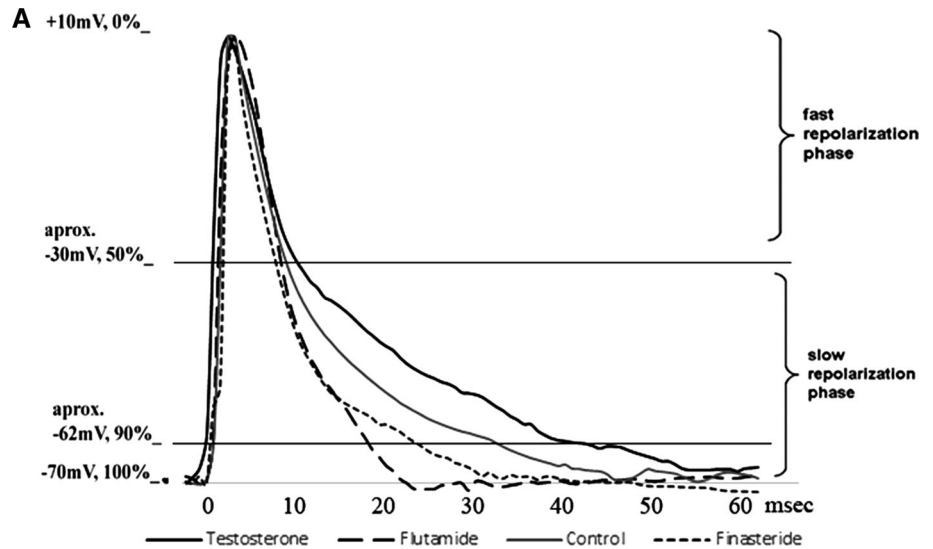
	TTP	APD50	APD90	QT
Control	2.9±0.14	8.78±0.4	28.51±2.15	63.6±3.4
Testosterone	3.09±0.11	10.28±0.76	44.32±2.41*	62.6±4.2
Flutamide	3.62±0.11*	8.61±0.28	21.35±0.72*	76.5±1.68*
Finasteride	3.6±0.15*	10.27±0.26	30.57±1.15	75.0±1.1*
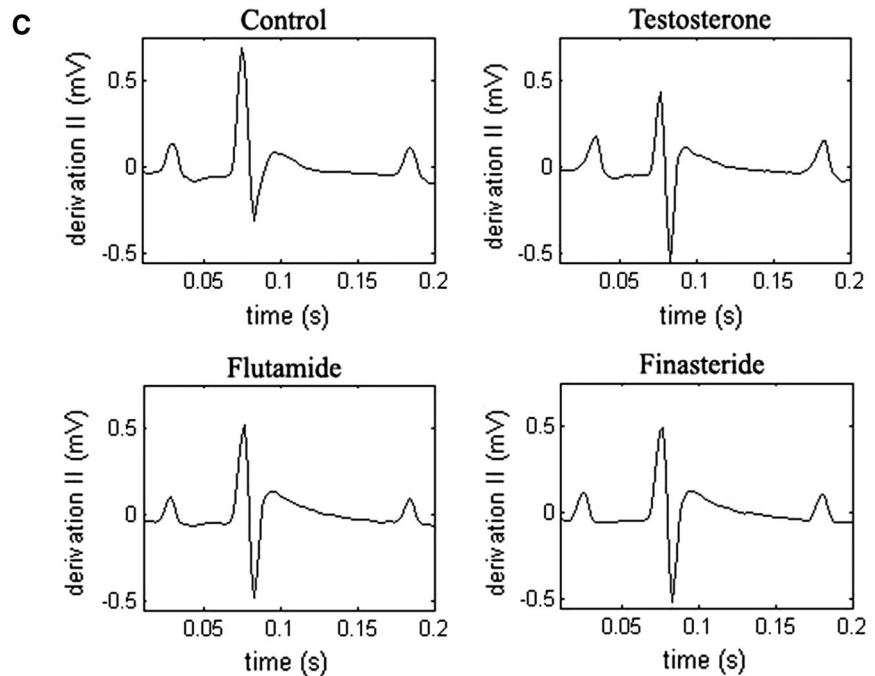
the four groups (control, testosterone, finasteride, and flutamide). At the mRNA level, there was a 43.9 ± 11.9 % increase in the expression of NCX1 in the testosterone-treated rats compared to the control, which is a significant difference ($p < 0.05$) (Fig. 4d), but the testosterone treatment failed to cause a significant change in the expression of the voltage-gated potassium channel KCND3 (Fig. 4a). Compared to the control, the finasteride and flutamide treatments caused an inhibition of KCND3 expression of 33.8 ± 4.1 and 43.0 ± 8.9 % (both
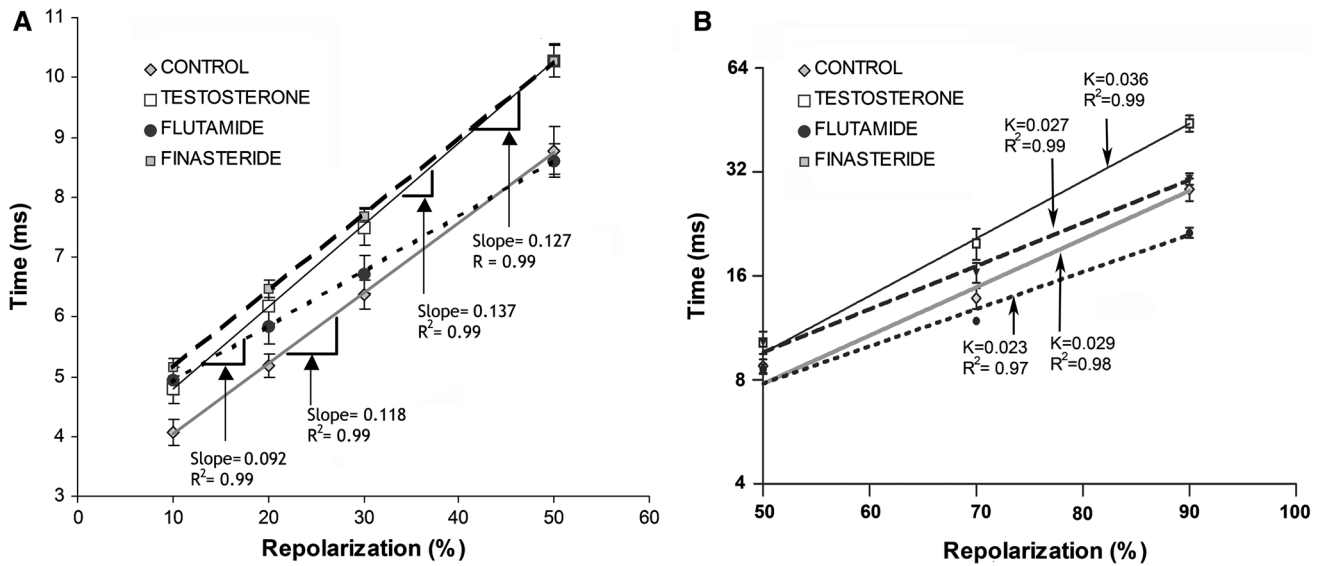
Fig. 3 Minimum square regression analysis. **a** Plot of the linear regression for the fast repolarizing phase for each rat group ($n = 8$). **b** Plot of the exponential regression for the slow repolarizing phase for each rat group. $n = 8$ rats per group
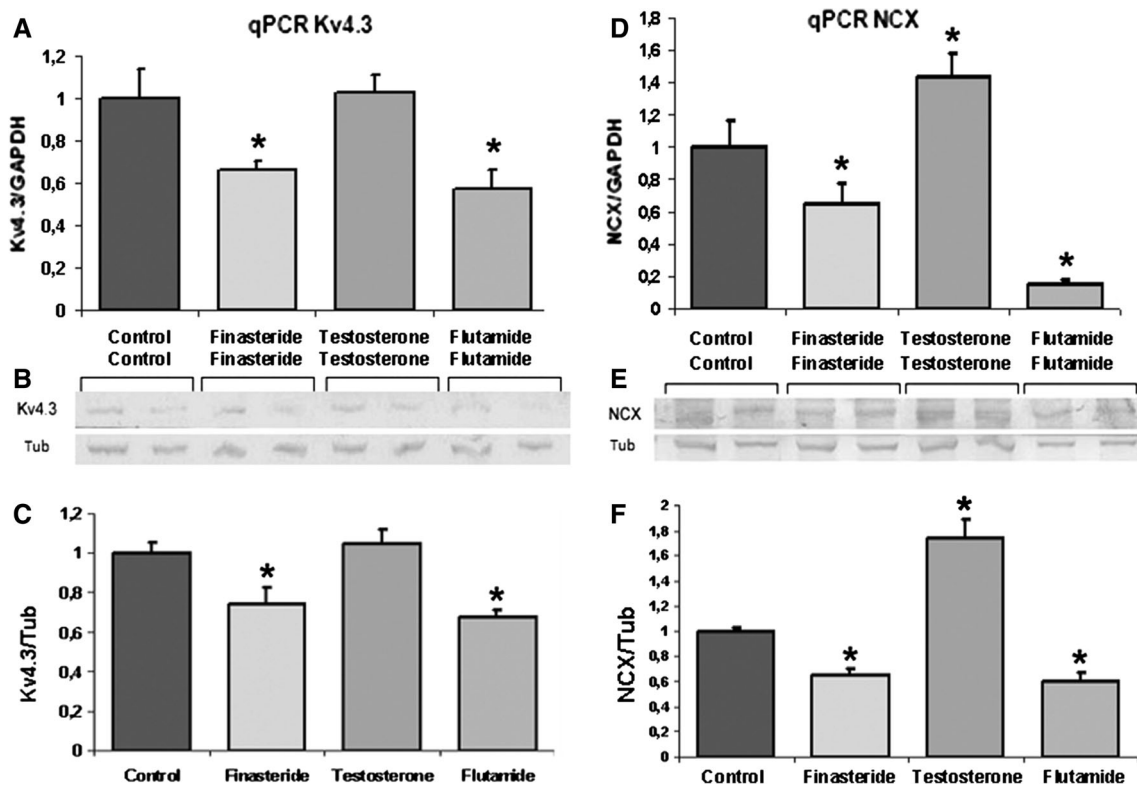

Fig. 4 Control of expression of the voltage-gated potassium channel Kv4.3 and the bi-directional membrane ion transporter NCX in the rat right ventricle. **a, d** mRNA levels of Kv4.3 (**a**) and NCX (**d**) by real time PCR semi-quantitative (*q*) analysis normalized to glyceralde-hyde 3-phosphate dehydrogenase (*GAPDH*) expression. mRNA level is given as the mean ± standard error of the mean (SEM). *Asterisk* Significantly different from control at $p < 0.05$. $n = 6$ rats per group.

b, e Representative western blots comparing Kv4.3 (**b**) and NCX (**e**) expression in the control and finasteride-, testosterone-, and flutamide-treated right ventricular lysates. **c, f** Quantification of three replicate immunoblots for Kv4.3 (**c**) and NCX (**d**). Protein level is given as mean ± SEM related to the housekeeping β-tubulin (*Tub*). *Asterisk* Significantly different from control at $p < 0.05$. $n = 5$ rats per group

$p < 0.05$), respectively (Fig. 4a) and an inhibition of NCX1 expression by 34.4 ± 12.1 and 84.7 ± 2.3 % (both $p < 0.05$), respectively (Fig. 4d). Western blot analysis of NCX and Kv4.3 expression revealed that testosterone did not significantly change the expression of Kv4.3 (Fig. 4b, c), but it did increase that of NCX (Fig. 2e, f) by 74.3 ± 14.5 % ($p < 0.05$). Compared to the control, finasteride inhibited Kv4.3 expression by 26.1 ± 8.7 % ($p < 0.05$) (Fig. 4b, c) and NCX expression by 34.3 ± 4.7 % ($p < 0.05$) (Fig. 2e, f), while flutamide inhibited kv4.3 expression by 32.3 ± 4 % ($p < 0.05$) (Fig. 4b, c) and NCX expression by 39.2 ± 6.5 % ($p < 0.05$) (Fig. 2e, f).

Discussion

Research involving steroid hormones is associated with a number of difficulties. Interconnected metabolic and biosynthetic pathways usually complicate the interpretation of experimental results [24, 25]. In addition, at least in the case of the AR, the effects observed can be due to genomic or non-genomic responses [26, 27]. In our experimental design we attempted to tackle these challenges by having two points of androgenic inhibition that differed both in nature and intensity.

Finasteride is an inhibitor of 5-α-reductase, the enzyme which converts testosterone into dihydrotestosterone (DHT) [28]. DHT has a higher affinity for AR than testosterone [29]. Consequently, finasteride indirectly decreases, at least partially, the activity of the AR at the transcriptional level and that resulting from non-genomic responses. In comparison, flutamide is a competitive inhibitor of the AR, preventing its transcriptional activity, but it does not inhibit the non-genomic effects of androgens that are independent of the AR [26].

Androgens have different effects on each phase of the rat cardiac AP. On phase 0 testosterone supplementation has no detectable effect on phase 0, nevertheless both finasteride and flutamide treatment resulted in increased TTP. Interestingly, despite the longer APD90 in the control and testosterone groups, QT intervals for these two groups were shorter than those of the flutamide and finasteride groups. This is possibly the result of the smaller slope in phase 0 ($\partial V / \partial t$) of the latter two groups, since the slope of phase 0 is one of the main determinants of conduction velocity [30], and conduction velocity is inversely related to the duration of the QT interval [31]. In addition, QT duration is directly related to APD90 [31]. In most naturally occurring phenomena (e.g. autonomic nervous system stimulation), an increase in conduction velocity is associated with a shorter APD90 or vice versa. In these cases both components (APD and conduction velocity) work in the same direction—whether to increase or decrease QT duration. Nevertheless, conduction velocity and APD90 are independent phenomena. Therefore, a situation is conceivable in which conduction is slower, thereby increasing QT duration even with a shorter APD90. Conduction velocity is dependent on Ina and is inversely proportional to QT duration [31]. Drugs such as terfenadine inhibit the voltage-gated Na^+ channel Nav1.5 and decrease dV/dt without changing APD90 [32]; other drugs, such as indapamide, also modify QT duration [33] without modifying the APD90 [34]. Based on the results of our model we propose that in the rat heart flutamide can decrease the conduction velocity (as a result of longer TTP), while decreasing APD90. The outcome, if this is the case, would be a longer QT because the effect of the slower conduction outweighs the shorter APD90.

Under our experimental conditions, the slope of the fast repolarization phase did not differ significantly among the groups, leading to the conclusion that androgens do not participate in the control of this phase. Testosterone supplementation did not lead to Kv4.3 regulation. However, despite this result and in agreement with previous data indicating androgenic control of Kv4.3 in dogs [35], we observed a clear inhibition of Kv4.3 expression by both the finasteride and flutamide treatment. Considering that Kv4.3 is a determining factor for Ito intensity [14, 15, 36], it is unclear why this change in Kv4.3 abundance was not reflected in a slower repolarization.

The slow repolarization phase is clearly regulated by androgenic activity. The range of values for APD90 went from 21.35 ms for the flutamide-treated group to 44.32 ms for the testosterone-supplemented group, despite there being no significant differences between the two groups at APD50. We observed that the slopes of the different groups were ordered in direct relation with androgenic activity, with significant differences between the testosterone-treated group and the finasteride- or flutamide-treated group, clearly showing an incremental effect that correlated with androgenic activity.

An analysis of the literature supports the notion that most if not all of the changes observed can be attributed to androgenic regulation of NCX1 expression. Although downregulation of NCX1 can be compensated [37], isolated cardiac myocytes from transgenic mice models overexpressing NCX1 result in a 50 % increase in APD90 with no changes in APD50 [18].

In our model, arrhythmogenicity was clearly dependent on androgenic activity. APPs resulted in VEAP 30 % of the time in the right ventricle of the control rats. Testosterone supplementation caused this frequency to increase to a 50 % of positive APPs. In comparison, only 11 % of the APPs in the finasteride group and 17 % of the APPs in the flutamide group were positive. Working in a model of

NCX1 overexpression in mice Pott and colleagues found that higher NCX expression results in early afterdepolarization (EADs) and delayed afterdepolarization (DADs), while they found no such events in wild-type mice [18]. Our model clearly differs from that of Pott et al. [18] in terms of species (mice vs. rat), NCX fold variation (20 vs. 3), and Langendorff preparation (whole heart vs. right ventricle). Nevertheless, the similarity of the results of these two studies suggests a common mechanism related to a higher activity of NCX working in forward mode at or near resting membrane potential.

Conclusions

In summary, contrary to the situation in most mammals androgens lengthen rat cardiac AP. This lengthening appears to be a direct consequence of the specific set of currents present—and more importantly absent—in the rat heart. With the rat having a much weaker I_{k1} than comparable animals, such as guinea pigs or rabbits, final repolarization of the rat AP is much slower and, therefore, more sensitive to changes in a depolarizing current, such as that of NCX at this stage of the AP [17]. We found that NCX1 expression is induced by androgens, resulting in an even slower final repolarization. Also, contrary to other mammal models, such as rabbits [38] and guinea pigs [39], in rats, testosterone has pro-arrhythmogenic effects, possibly directly due to higher NCX expression facilitating the triggering of DADs from spontaneous Ca^{++} release or oscillatory events [40]. Another possible mechanism indirectly related to increased NCX expression could be the occurrence of EADs due to delayed repolarization.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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