

Testosterone Causes Simultaneous Decrease of $[Ca^{2+}]_i$ and Tension in Rabbit Coronary Arteries: by Opening Voltage Dependent Potassium Channels

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The relationship between the level of testosterone and the incidence of coronary heart disease is still controversial in the view of the results of clinical and epidemiologic studies. This uncertainty might be partly due to relatively small number of experimental studies undertaken to investigate the cellular mechanism underlying the vascular responses to testosterone. To further investigate the cellular mechanisms of testosterone with respect to vascular response, we investigated the effect of testosterone on contractility and intracellular Ca^{2+} regulation in a rabbit coronary artery and evaluated the underlying mechanism of testosterone-induced changes of coronary vascular tone by using various pharmacological blockers.

Testosterone was found to relax rabbit coronary arteries in a dose-dependent manner, and no significant difference was found in the relaxation response to testosterone with or without endothelium. Similar results were obtained in male and non-pregnant female rabbit coronary arteries. The relaxation response of rabbit coronary arteries to testosterone was greater for PGF2 α -contracted rings than for KCl contracted rings, which suggest the involvement of K^+ channels. Furthermore, the relaxation response to testosterone was significantly reduced by 4-aminopyridine, a sensitive blocker of voltage dependent K^+ channels, but not by low doses of tetraethylammonium or iberiotoxin, a Ca^{2+} activated K^+ channel blocker. Testosterone simultaneously reduced the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and tension, and 4-AP effectively antagonized the testosterone-induced change of $[Ca^{2+}]_i$ and tension. Therefore, it may be concluded that the stimulation

of voltage dependent K channels is responsible, at least in part, for the testosterone-induced relaxation of rabbit coronary arteries.

Key Words: Testosterone, voltage dependent K^+ channels, rabbit coronary artery, 4-aminopyridine, intracellular Ca^{2+}

INTRODUCTION

It is generally accepted that the incidence of morbidity and mortality from coronary artery disease (CAD) depends on both gender and age. For example, the risk of developing CAD is less for premenopausal women than for men or postmenopausal women.^{1,2} Many epidemiologic and experimental studies have proposed that estrogen may protect from the development of CAD via its lipid-lowering and antioxidant effects.³ Testosterone, on the other hand, is often considered to exacerbate the development of CAD, because the incidence of CAD is higher in men, and some studies have linked the level of plasma testosterone with the incidence of CAD.^{4,5} However, many other studies have reported that testosterone inhibits CAD development. For example, testosterone has been associated with a higher level of high density lipoprotein in men, which is negatively correlated with development of CAD, in addition, risk factors for CAD, such as fibrinogen, plasminogen activator inhibitor-1 have also been found to be negatively correlated with serum testosterone levels.⁶ Furthermore, hypotestosteronemia is associated with myocardial infarction in men,⁷ and testosterone was found to relieve

Received September 26, 2003

Accepted October 14, 2003

This study was supported by a faculty research grant from the Yonsei University College of Medicine for 1999 (No. 1999-02).

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exercise induced depression of the ST segment by electrocardiography.⁸ In the light of these clinical and epidemiological studies, the relationship between testosterone and CAD remains controversial, and yet more study is required to better understand the mechanistic relation between testosterone and CAD.

Recently, it was reported that the *in vitro* application of testosterone produces acute coronary artery relaxation, and suggested that this due in part to changes in K^+ flux or Ca^{2+} flux across cell membrane.^{9,10} Moreover, testosterone-induced relaxation response was antagonized by glibenclamide, tetraethylammonium, or 4-aminopyridine in canine, porcine, and rat arteries.¹⁰⁻¹² These reports suggest that the potassium channels, stimulated by testosterone, may be heterogenous with respect to the type of artery or species. Furthermore, it remains uncertain whether increased K^+ efflux by testosterone is correlated with attenuated $[Ca^{2+}]_i$ and decreased tension. Thus, the primary goal of present study was to identify K^+ channel type involved in testosterone-induced coronary relaxation by measuring vascular tone in the presence of various blockers. We then investigated the effect of testosterone on $[Ca^{2+}]_i$. The finding of this study provide evidence that 4-aminopyridine sensitive, voltage dependent K^+ channels may be responsible for testosterone-induced relaxation and attenuation of $[Ca^{2+}]_i$ in the rabbit coronary artery smooth muscle cells.

MATERIALS AND METHODS

Tissue preparation and tension measurements

After anesthetizing a rabbit with pentobarbital sodium (60 mg/Kg), the heart was extracted and placed in a preparation chamber containing a physiological salt solution (PSS). In the preparation chamber, coronary arteries were meticulously dissected from neighboring tissues with an ophthalmologic scissors and forceps. Coronary artery rings were suspended in a organ bath under a resting tension of 0.8 g. After being allowed to equilibrate for 1 hr in PSS, each ring was repeatedly exposed to 80 mM KCl solution until the contracting responses became stable.

The 80 mM KCl solution was prepared by replacing NaCl with equimolar KCl in PSS. Muscle contractive force was determined with a force-displacement transducer and recorded on a computer.

Fura-2 loading and simultaneous measurement of tension and $[Ca^{2+}]_i$

Intracellular Ca^{2+} levels ($[Ca^{2+}]_i$) and force of muscle contraction were measured as described by Kwon, et al.¹³ Muscle strips were exposed to acethoxymethyl ester of fura-2 (fura-2/AM, 5 μ M) in the presence of 0.01% cremophore EL for 5 ~ 6 hr at room temperature (22 - 24°C). A coronary strip was placed in an experimental chamber, and then illuminated alternatively (48 Hz) with 340 nm and 380 nm light. The ratio of the 500 nm fluorescence induced by 340 nm excitation (F340) over that induced by 380 nm excitation (F380) was determined using a spectrophotometer (CAF 110, Japan Spectroscopic, Tokyo, Japan). The value of the ratio for 70 mM KCl was taken as the reference response (100%).

Solutions and chemicals

PSS had following composition (mM): NaCl 118; KCl 4.8; $CaCl_2$ 2.5; KH_2PO_4 1.2; $MgSO_4$ 1.5; $NaHCO_3$ 25; and Glucose 11. PSS was saturated with a mixture of 95% O_2 and 5% CO_2 , and adjusted to pH 7.4. The temperature was maintained at 37°C. Testosterone, TEA, and 4-AP were purchased from the Sigma Chemical Co. All drugs were prepared on the day of the experiment. Stock solutions of testosterone was dissolved in dimethylsulfoxide to a testosterone concentration of 10 mM and then further diluted with PSS solution.

Statistics

Results are expressed as means \pm S.E.M. Student's t-test was used for the statistical analysis, and the number of preparation taken from separate animals was indicated by n. *p* values of less than 0.05 were considered to be significant.

RESULTS

Effect of endothelium on testosterone-induced relaxation in rabbit coronary artery

Testosterone produced a concentration-dependent relaxation of coronary arterial rings precontracted with $\text{PGF}_2\alpha$. The concentration-response relationship for testosterone-induced relaxation of the endothelium (EC)-intact coronary rings is shown in Fig. 1A. The involvement of endothelium in mediating testosterone-induced relaxation was investigated by obtaining a series of concentration relaxation responses in EC-denuded coronary rings (Fig. 1B). The removal of EC resulted in a slight, but insignificant shift in the testosterone response curve (EC_{50} values; $3.6 \pm 0.1 \mu\text{M}$ in EC intact rings; $2.8 \pm 0.2 \mu\text{M}$ in EC-denuded rings; $n=20$).

The involvement of gender was also examined by comparing the effect of testosterone in both sexes. As shown in Fig. 2, no significant difference was observed between the male and female coronary ring testosterone response curves. The sensitivity (EC_{50} values) to testosterone-induced relaxation in male coronary rings was $2.6 \pm 0.1 \mu\text{M}$ ($n=12$), whilst in female coronary rings, the EC_{50} values insignificantly changed to $3.0 \pm 0.2 \mu\text{M}$ ($n=12$, $p>0.05$).

Involvement of K^+ channels in testosterone-induced relaxation

At a normal external K^+ concentration (5 mM K^+), coronary rings precontracted with $\text{PGF}_2\alpha$

relaxed $88.4 \pm 1.6\%$ ($n=8$) in response to $10 \mu\text{M}$ testosterone. In contrast, the same concentration of testosterone produced a relaxation of $26.6 \pm 3.0\%$ ($n=8$) in the same coronary rings precontracted in a 80 mM K^+ solution (Fig. 3.) These findings indicate that the trans-sarcolemmal K^+ gradient for K^+ efflux is critically required for the testosterone effect and suggest the involvement of K^+ channels in testosterone-induced rabbit coronary ring relaxation.

Pretreating coronary arteries with a low dose (1 mM) of tetraethylammonium (TEA) slightly alleviated the testosterone-induced relaxation responses. At this low concentration, TEA is known to selectively block large conductance Ca^{2+} -activated K^+ channel (BK_{Ca}) in smooth muscle cells.¹⁴ The magnitudes of the relaxations induced by testosterone ($10 \mu\text{M}$) were $89.9 \pm 1.7\%$ and $87.4 \pm 5.9\%$ before and after TEA pretreatment, respectively ($n=10$). Pretreatment with 10 nM iberiotoxin, a highly selectively blocker of BK_{Ca} , had a similar effect as TEA pretreatment on testosterone-induced relaxation, i.e., $88.2 \pm 3.2\%$ and $85.2 \pm 1.9\%$ before and after iberiotoxin pretreatment, respectively; $n=10$. These results suggest that the BK_{Ca} channel might not be a target for testosterone-induced relaxation in rabbit coronary arteries.

Pretreating coronary arteries with 5 mM 4-aminopyridine (4-AP), a blocker of voltage dependent K^+ channel, significantly attenuated testosterone-induced relaxation response. The magnitude of relaxation by testosterone ($10 \mu\text{M}$) was $89.9 \pm 1.7\%$ and $27.2 \pm 3.8\%$ with and without 4-AP pre-

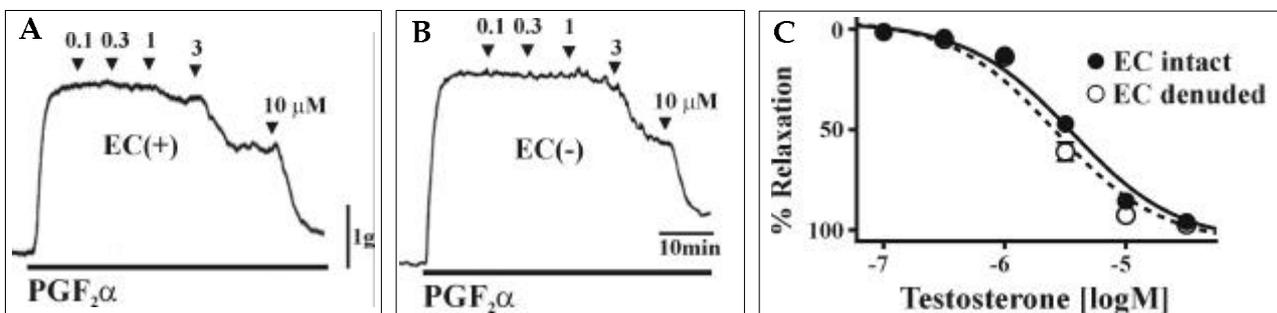


Fig. 1. Effect of endothelium on testosterone-induced relaxation responses. Typical recordings of the inhibitory effect of testosterone on $3 \mu\text{M}$ $\text{PGF}_2\alpha$ -induced contraction in the presence (A) and in the absence of endothelium (B). (C) Summarized results of testosterone-induced relaxation responses with or without endothelium in pooled male and female coronary ring data. The degree of relaxation was expressed as a percentage of $\text{PGF}_2\alpha$ contraction. Values are expressed as means \pm SEM ($n=21$). The EC_{50} value was $3.6 \pm 0.1 \mu\text{M}$ vs. $2.8 \pm 0.2 \mu\text{M}$ in endothelium-intact or -denuded rings; $n=20$, $p>0.05$).

treatment, respectively (n=10).

Furthermore, the application of 4-AP reversed testosterone-induced $[Ca^{2+}]_i$ attenuation in rabbit coronary strips. As shown in Fig. 5, testosterone simultaneously attenuated $[Ca^{2+}]_i$ and decreased tension in rabbit coronary strips, and the application of 1 mM 4-AP in the presence of testosterone increased the tension and reduced $[Ca^{2+}]_i$ to

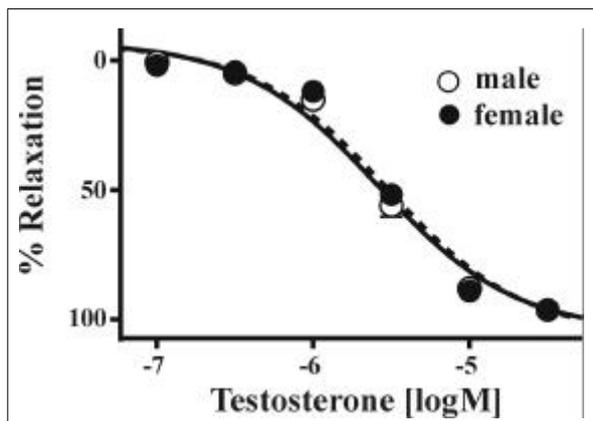


Fig. 2. Effect of gender on testosterone-induced relaxation responses. Summarized results of testosterone relaxation responses in endothelium-denuded male and female coronary arteries (EC_{50} values; $2.6 \pm 0.1 \mu M$ in males; $3.0 \pm 0.2 \mu M$ in female, $n=12$, $p>0.05$).

the control level (n=5).

DISCUSSION

The present study demonstrates that in rabbit coronary arteries testosterone has a relaxing effect on smooth muscle cells. No difference was seen between arteries from male and female rabbits. Moreover, testosterone-induced responses required physiological gradients of potassium, suggesting K^+ channel involvement. Subsequent studies using various K^+ channel blockers provided evidence that testosterone activates the activity of voltage dependent K^+ channels (K_v) in coronary smooth muscle cells. Testosterone simultaneously reduced $[Ca^{2+}]_i$ and tension, and 4-AP effectively antagonized testosterone-induced these $[Ca^{2+}]_i$ and tension changes in rabbit coronary strips. Therefore, we conclude that stimulation of the K_v channel might be primarily responsible for testosterone-induced relaxation in rabbit coronary arteries.

Recent studies have reported that testosterone relaxes rat, rabbit, porcine, and canine coronary arteries and aortas.^{10-12,15,16} In the rat aorta and the

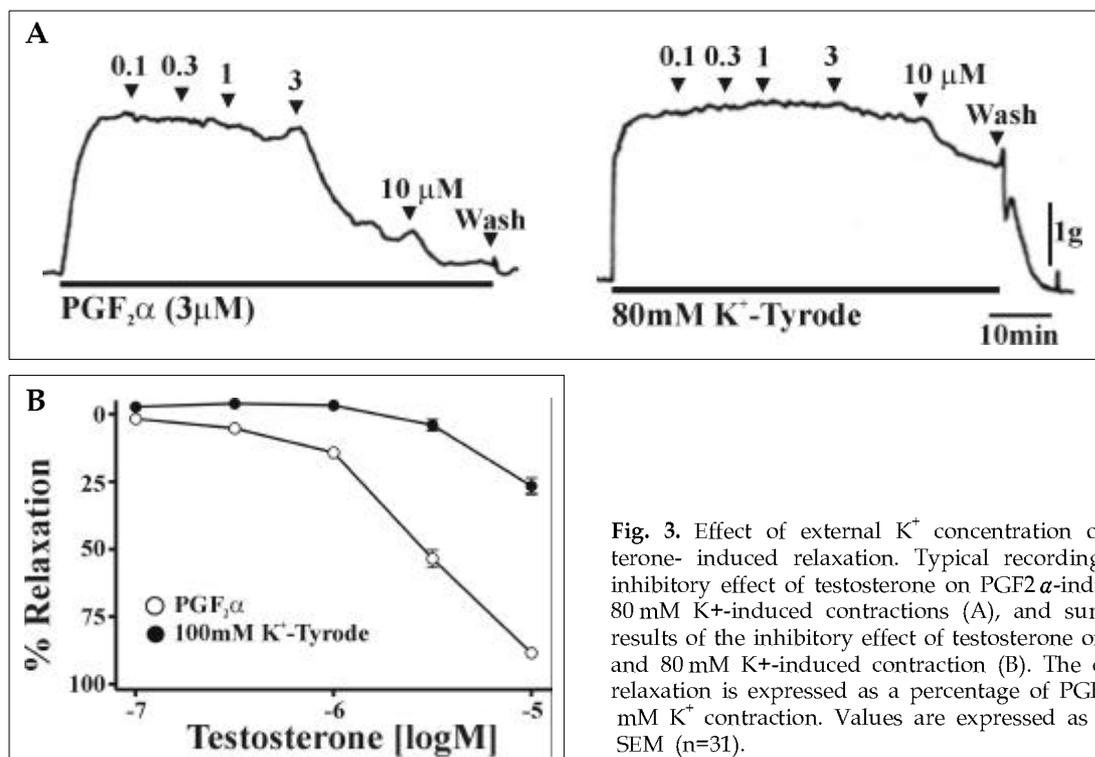


Fig. 3. Effect of external K^+ concentration on testosterone-induced relaxation. Typical recordings of the inhibitory effect of testosterone on $PGF_2\alpha$ -induced and 80 mM K^+ -induced contractions (A), and summarized results of the inhibitory effect of testosterone on $PGF_2\alpha$ and 80 mM K^+ -induced contraction (B). The degree of relaxation is expressed as a percentage of $PGF_2\alpha$ or 80 mM K^+ contraction. Values are expressed as means \pm SEM (n=31).

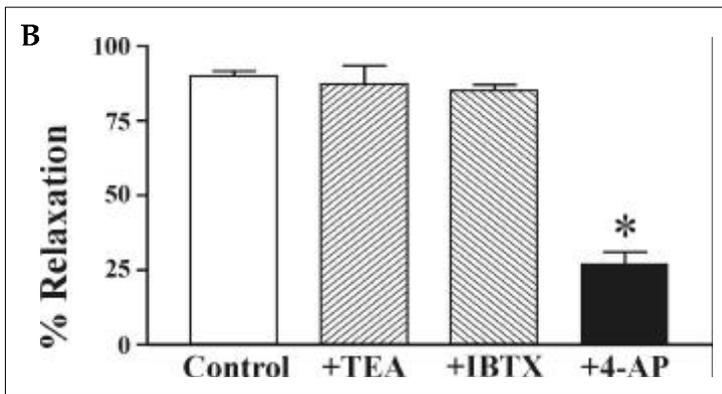
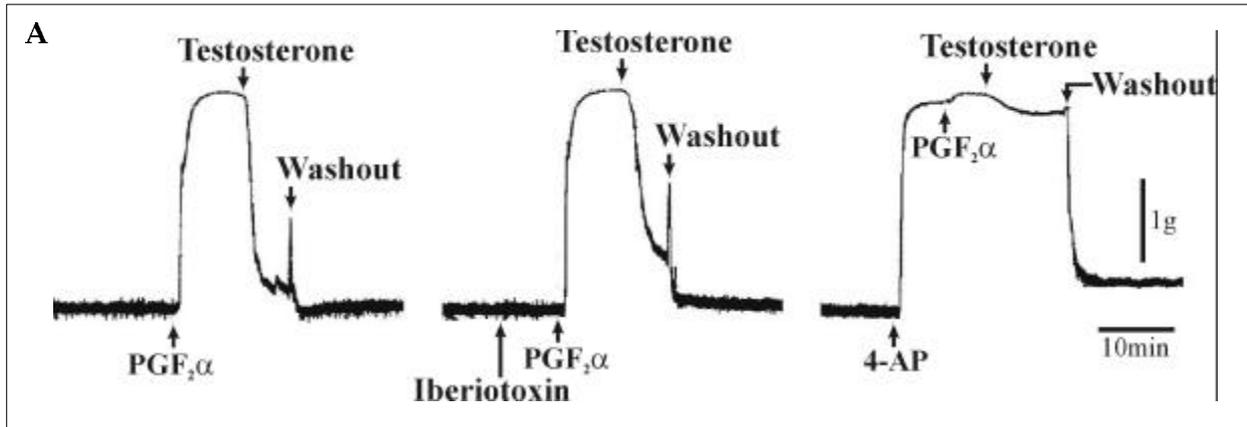


Fig. 4. Effect of pretreatment of 4-AP, TEA, or iberiotoxin on testosterone-induced relaxation responses. (A) Typical recordings of inhibitory effect of K^+ channel blockers on $10\ \mu\text{M}$ testosterone-induced relaxation responses. (B) Summarized results of percentage of relaxation by $10\ \mu\text{M}$ testosterone in the control (open bar), after treating with $1\ \text{mM}$ TEA (right hatched bar), $10\ \text{nM}$ iberiotoxin (IBTX) (left hatched bar), or $5\ \text{mM}$ 4-AP (filled bar). The degree of relaxation was expressed as a percentage of $\text{PGF}_2\ \alpha$ contraction. Values are expressed as means \pm SEM ($n=10$). Asterisk represents $p<0.01$.

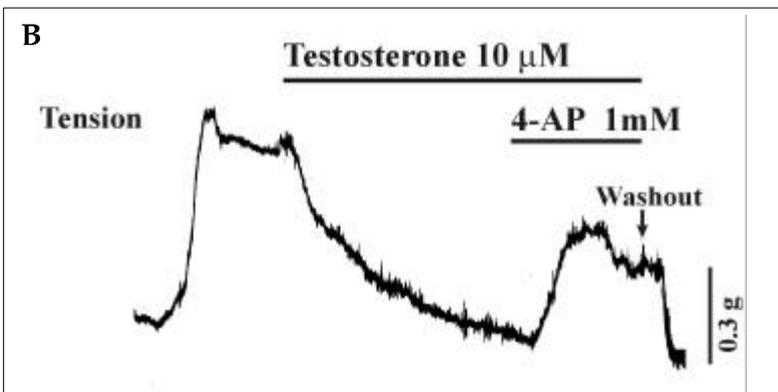
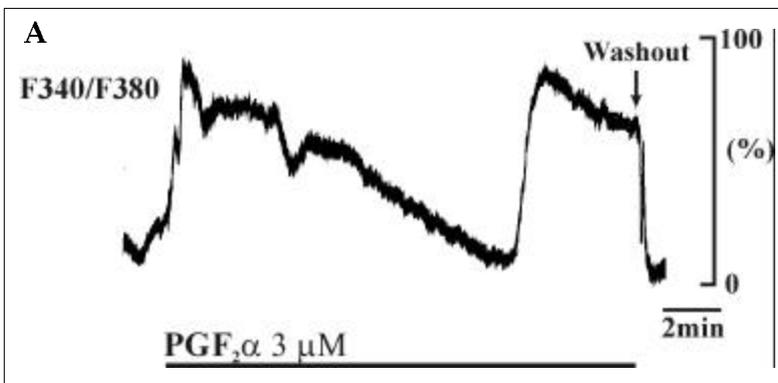


Fig. 5. Effect of 4-AP on testosterone-induced change of $[\text{Ca}^{2+}]_i$ and tension in rabbit coronary strips. Testosterone-induced attenuation of $[\text{Ca}^{2+}]_i$ attenuation (A) and relaxation of contraction (B) in coronary strips precontracted with $\text{PGF}_2\ \alpha$. Fluorescence ratio (F340/F380) was used to determine $[\text{Ca}^{2+}]_i$ and expressed as a percentage of the fluorescence ratio in $70\ \text{mM}$ KCl. Application of $1\ \text{mM}$ 4-AP completely reversed testosterone-induced $[\text{Ca}^{2+}]_i$ attenuation.

canine coronary artery, testosterone relaxed arterial tone partly by enhancing NO release from the endothelium,^{11,15} and in the rabbit aorta and porcine coronary artery, testosterone induced endothelium-independent relaxation.^{10,16} In the present study, using rabbit coronary artery, the removal of the endothelium was not found to significantly affect testosterone-induced relaxation. Furthermore, pretreatment with L-NAME, an inhibitor of NO synthesis, did not affect relaxation responses by testosterone (unpresented result). Therefore, it is likely that the primary target of testosterone is smooth muscle cells, and not the endothelium in the rabbit coronary artery.

The contractile force of arterial smooth muscle depends on $[Ca^{2+}]_i$ and Ca^{2+} influx through Ca^{2+} channels is one of the major determinants of $[Ca^{2+}]_i$.¹⁴ Moreover, Ca^{2+} entry through Ca^{2+} channels is proportional to the amount of time a Ca^{2+} channel spends in the open state, which is strongly regulated by membrane potential (E_m) of smooth muscle cells.¹⁴ In vascular smooth muscle cells, E_m is mainly controlled by K^+ flux through sarcolemmal K^+ channels, and the amount of K^+ flux depends on both the number of K^+ channels and the electrochemical gradient of potassium across the membrane.¹⁷ If the electrochemical gradient for K^+ is diminished in high external K^+ solution, the change in the number of K^+ channels by some agonist has little effect on E_m , and on Ca^{2+} influx. In the present study, testosterone caused a significant decrease in both tension and $[Ca^{2+}]_i$ level at physiologic K^+ levels, and these testosterone-induced relaxation responses were significantly attenuated in arteries pre-contracted with 80 mM KCl. These findings indicate that testosterone-induced relaxation response requires suitable potassium gradients for K^+ efflux, and suggest the activation of K^+ channels by testosterone. Previous studies reported that BaCl, a non-selective blocker of K^+ channel, profoundly reduces testosterone-induced relaxation responses.¹⁶ These findings are consistent with those of the present study, and further suggest the testosterone-induced activation of K^+ channels. However, the type of K^+ channels affected by testosterone remains uncertain.

In rabbit coronary smooth muscle cells, at least four types of K^+ channels have been identified:

voltage-dependent K^+ channels (K_v), Ca^{2+} -activated K^+ channels (BK_{Ca}), ATP-sensitive K^+ channels (K^{ATP}), and inward rectifier K^+ channels (I_{K1}).^{14,17-19} Of these K^+ channels, BK_{Ca} and K_v channels are considered important effector systems, which mediate agonist-induced changes of vascular tone by altering the membrane potential of smooth muscle cells.^{14,19}

Deenadayalu, et al.¹⁰ reported that the inhibition of BK_{Ca} by low doses of TEA or iberiotoxin, significantly increased the resting tone of porcine coronary artery, which suggests the importance of BK_{Ca} in the setting and maintaining the resting E_m under physiologic conditions. Moreover, testosterone-induced relaxation was effectively antagonized by iberiotoxin or TEA, suggesting the importance of BK_{Ca} channels in the regulation of tension under resting or stimulated conditions, and indicating that these channels are a principal target for testosterone-induced relaxation in the porcine coronary artery. However, in our present study the inhibition of BK_{Ca} by 1 mM TEA or iberiotoxin had no significant effect on the resting tension or testosterone-induced relaxation. These findings suggest that in the rabbit coronary artery BK_{Ca} does not play an important role in the regulation of resting tone, and is not a target for testosterone-induced relaxation. Similar findings were also reported in the rat aorta, in which TEA had no effect on testosterone-induced relaxation. These reports suggest that the nature of potassium channels stimulated by testosterone may be heterogenous with respect to the type of artery and species. This hypothesis is further supported by the more than 10 fold difference in the effective concentration of testosterone (EC_{50} value) between our present results ($2.8 \pm 0.2 \mu M$) and previous reports ($44.4 \pm 9.7 \mu M$ in a porcine coronary artery, and more larger $100 \mu M$ in a rabbit aorta).^{10,16}

In the rabbit coronary artery it has been reported that K^+ flux through K_v channels is responsible for determining resting E_m , and that the inhibition of K_v channels by some agents induces the contraction of rabbit coronary arteries.¹⁹⁻²¹ These reports suggest the importance of K_v channels in the regulation of vascular tone in the rabbit coronary artery under either stimulated or unstimulated conditions. As shown in Fig. 4, the

application of 4-AP, a sensitive K_v channel blocker, induced the contraction of unstimulated rabbit coronary arteries and significantly inhibited testosterone-induced relaxation responses. The involvement of the K_v channel in testosterone-induced relaxation was also reported in the rat aorta, in which the application of 4-AP significantly attenuated testosterone responses.¹² Moreover, testosterone-induced $[Ca^{2+}]_i$ attenuation was also reversed by 4-AP treatment. These results suggest that the potassium channels stimulated by testosterone in rabbit coronary arteries are K_v channels.

In summary, we report that testosterone induces the endothelium-independent relaxation of rabbit coronary artery. Moreover, tension and intracellular Ca^{2+} measurements indicate that this response primarily involves the stimulation of K_v channels. However, the transduction mechanisms from the activation of the testosterone receptor to the activation of K_v channels remain to be elucidated, and further direct molecular evidence is required to prove that testosterone opens the K_v channel in rabbit coronary smooth muscle cells.

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