Investigation of testosterone-mediated non-transcriptional inhibition of Ca^{2+} in vascular smooth muscle cells

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Abstract. The aim of the present study was to observe the effect of short-term testosterone treatment on Ca^{2+} in vascular smooth muscle cells (VSMCs) of male rats. Cells were loaded with the Ca^{2+}-sensitive fluorescent indicator Fura-2 and intracellular Ca^{2+} signals of VSMCs were measured using a Nikon TE2000-E live cell imaging workstation. The baseline level of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in resting state VSMCs was ~100 nmol/l. Testosterone alone led to a slow increase in $[Ca^{2+}]_i$, but there was no significant difference compared with the ethanol vehicle control. When VSMCs were stimulated with a high-potassium solution (containing 42 mmol/l of K^+), $[Ca^{2+}]_i$ rose rapidly and remained at a high plateau level. Short-term treatment using physiological (40 nmol/l) or supraphysiological (4 μmol/l) levels of testosterone at either the plateau phase or the pretreatment stage could significantly inhibit the $[Ca^{2+}]_i$ increase induced by high-potassium solutions. Testosterone coupled to bovine serum albumin also had a similar effect and repetitive testosterone interventions over a short time-frame led to inhibition. Testosterone has a non-transcriptional inhibition effect on the $[Ca^{2+}]_i$ of VSMCs and acts with the cell membranes of VSMCs to inhibit voltage-gated Ca^{2+} channel-mediated Ca^{2+} influx, which may be one of the mechanisms underlying testosterone-mediated vasodilation.

Introduction

The incidence of coronary heart disease differs by gender (1,2). In recent years, the functions of androgen in the pathogenesis of coronary heart disease in males have attracted increasing attention from investigators (3,4). Clinical trials, animal experiments and *in vitro* studies all strongly suggest that androgen plays an important role in regulating vascular function.

Androgens belong to the steroid family. The classical functional pathway of androgens involves binding to the intracellular androgen receptors (AR) and entering the nucleus as transcription factors to regulate target gene transcription, known as the genomic effect. In addition, non-genomic effects of androgens (also known as non-transcriptional effects) have also been frequently reported (5).

Androgens regulate vascular function at the transcriptional and non-transcriptional levels. Testosterone can cause rapid vasodilation by non-genomic mechanisms that primarily rely on an endothelium-independent pathway (6). The exact mechanism is unclear and the majority of existing studies are observations at the tissue level, whereas studies at the cellular and molecular levels are rarely reported. The present study used primary cultured rat aortic vascular smooth muscle cells (VSMCs) as the subject to investigate the regulation of testosterone on cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) and to demonstrate its non-transcriptional effects.

Materials and methods

Drug preparation. Testosterone was obtained from Acros Organics (Geel, Belgium) and was accurately weighed and dissolved in ethanol by stirring to create a $4x10^{-3}$ mol/l solution. This stock solution was further diluted with appropriate solutions to obtain the corresponding final concentrations immediately prior to use (the final concentrations of ethanol in various drug solutions were <0.1%). Testosterone coupled to bovine serum albumin (T-BSA) was purchased from Sigma (St. Louis, MO, USA); the molar ratio of testosterone and BSA was 23.6:1, the concentration of the stock solution was 800 μmol/l (800 μmol/l of T: 33.8 μmol/l of BSA) and that of the working solution was 400 nmol/l or 40 μmol/l.

Cell culture. The culture and identification methods of male Sprague-Dawley rat aortic VSMCs were as described in the previous study (7). Six days before drug intervention, regular fetal bovine serum (FBS) in the culture medium was replaced with FBS pre-treated with dextran-activated carbon to eliminate experimental interference due to sex hormones in the FBS. The cells within 10 generations were utilised in

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the experiments; toxicities such as apoptosis, shedding and reduced vigour were not observed during the experiment and cell viabilities were all >90%.

Experimental procedure. VSMCs in the logarithmic growth phase were transferred to culture dishes sealed in the bottom with a single layer of cover glass (sample window). When adherent cells reached <80% of confluence, cells were switched to serum-free medium and were maintained for 24 h to achieve synchronisation of the cell cycle [as the AR activity and level change with the cell cycle and are at their lowest in the G_1/S phase (8)]. The experiments used the Ca^{2+}-sensitive fluorescent indicator Fura-2 for the detection of intracellular Ca^{2+} signals in VSMCs.

Loading cells with Fura-2/AM. Fura-2 is membrane impermeable; therefore, it is difficult to load cells directly with Fura-2. However, Fura-2 and an acetyl-methyl group can form the lipid-soluble molecule Fura-2/AM via ester bonds, which can penetrate cell membranes and enter cells. Subsequently, Fura-2/AM can be hydrolysed by non-specific esterases inside cells to generate Fura-2; thus, loading of Ca^{2+}-sensitive fluorescent dye can be accomplished without damaging cells. As a non-ionic detergent with low toxicity, Pluronic F-127 can increase the water solubility of Fura-2/AM and can facilitate loading of cells. In the experiments, the loading solution consisted of Dulbecco's modified Eagle's medium (DMEM) with 2 μmol/l Fura-2/AM (Sigma) and 0.04% (w/v) Pluronic F-127 (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). Serum-free stationary VSMCs were added with the loading solution and maintained in the dark at 37˚C for 30 min. Cells were subsequently washed with HEPES-buffered saline [HBS; (pH 7.4) 10.0 mmol/l HEPES, 137 mmol/l NaCl, 5.0 mmol/l KCl, 1.0 mmol/l $Na_2HPO_4{\cdot}12\ H_2O$, 5.0 mmol/l glucose, 1.0 mmol/l $CaCl_2$ and 0.5 mmol/l $MgCl_2$], subjected to a de-esterification reaction for 30 min at room temperature in the dark and detected within 1 h.

Measurement of intracellular Ca^{2+} signalling. A Nikon TE2000-E live cell imaging system (Nikon, Tokyo, Japan) to measure the intracellular Ca^{2+} signal. The parameter settings were temperature at 30˚C, 40X objective lens and illuminance (neutral density) (8). The experiments used a three-dimensional hydraulic manipulator to micro-manipulate the pipetting needle close to cells in the observation vision field and used the 8-channel micro-perfusion system (ALA-VM8; ALA Scientific Instruments, Inc., Farmingdale, NY, USA) to replace the composition of the cell perfusate. The perfusion system used a pressure controller (PM 8000 Programmable Multichannel Pressure Injector; MicroData Instrument, Inc., South Plainfield, NJ, USA) to maintain the nitrogen pressure at 40 kPa. The high-speed scanning light source alternatively emitted monochromatic lights with a wavelength of 340 or 380 nm, which passed through the filter device and the fluorescence lens prior to being transmitted to cells in the sample window. The fluorescence emitted by Fura-2 loaded in cells was captured at the wavelength of 510 nm by the cooled charge-coupled device camera (C4742-80-12AG; Hamamatsu Photonics K.K., Hamamatsu, Japan). The sampling frequency of the fluorescent signal was 1 Hz, the image resolution was 512x512 pixels and the exposure times were 0.1 and 0.3 sec for red (380 nm) and green (340 nm), respectively. Switching of the excitation light wavelength, the camera shutter on/off and image data acquisition were controlled and completed using the software Simple PCI (v. 6.0; Compix Inc., Lake Oswego, OR, USA). Experimental data were converted to the excel format for offline processing. The measured baseline levels of the 340/380 nm fluorescence intensity ratio (Ratio) of VSMCs were between 1.0-1.2.

Calibration for intracellular Ca^{2+} concentration measurement. By detecting the fluorescence intensities at 340 and 380 nm excitation wavelengths, the free Ca^{2+} concentration was calculated using the Grynkiewicz formula (9):

$$\left[Ca^{2+}\right]_{free} = K_d \times \left[\frac{R - R_{\min}}{R_{\max} - R}\right] \times \left(\frac{F^{380}_{\max}}{F^{380}_{\min}}\right)$$

wherein K_d is the equilibrium dissociation constant of the binding between Fura-2 and Ca^{2+} and its value is 224 nm. R is the 340/380 nm Ratio, R_{min} is the value of R at zero Ca^{2+}, and R_{max} is the R value at saturated Ca^{2+}. F^{380}_{max} and F^{380}_{min} are the ratios of fluorescence intensity at 380 nm at zero intracellular Ca^{2+} and saturated Ca^{2+}, respectively. The above fluorescence intensity values were calculated after subtracting the background. As these parameters vary with the optical properties of the study system, zero Ca^{2+} solution (HBS containing 2.0 mmol/l EGTA and no $CaCl_2$), saturated Ca^{2+} solution (HBS containing 2.0 mmol/l $CaCl_2$) and the Ca^{2+} carrier ionomycin (5 μmol/l; Sigma) were used to artificially adjust the intracellular Ca^{2+} levels of VSMCs to the appropriate concentrations and subsequently calibrated the above parameters.

Results

Selection of cells for the imaging experiments. Ca^{2+} imaging showed that under the resting state, the baseline $[Ca^{2+}]_i$ level of VSMCs was ~100 nmol/l, consistent with previous reports in the literature (10). High concentrations of KCl solutions are commonly used to stimulate cell depolarisation, which can cause $[Ca^{2+}]_i$ to increase in VSMCs and can be used repeatedly. Therefore, the study first observed the reactivity of cells in the vision field to high-potassium solution (HBS containing 42 mmol/l K^+). Cells with strong reactions (i.e., $[Ca^{2+}]_i$ increased by >50%) and with stable baseline $[Ca^{2+}]_i$ levels were selected for further observation. Similar steps were performed for the following Ca^{2+} imaging experiments.

Effects of testosterone alone on the resting state $[Ca^{2+}]_i$ of VSMCs. The results of the effects of testosterone alone on the resting state of $[Ca^{2+}]_i$ in VSMCs are shown in Fig. 1A. VSMCs responded well to high-potassium stimulation. $[Ca^{2+}]_i$ increased rapidly from the baseline and was maintained at a high plateau level. Cells were subsequently perfused with blank HBS solution for 5 min so that the $[Ca^{2+}]_i$ level returned to baseline levels. Cells were again treated sequentially with physiological (40 nmol/l) and supraphysiological (4 μmol/l) concentrations of testosterone for 2 min (treated transitionally with HBS for 5 min between the two treatments), and the $[Ca^{2+}]_i$ was slowly elevated (<50 nM).

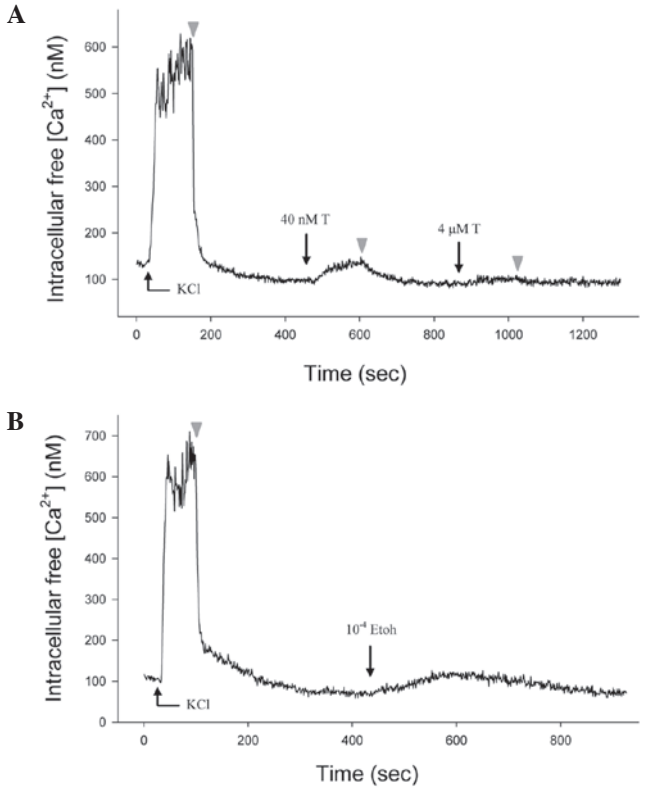

Figure 1. Effects of testosterone (T) and ethanol solvent on the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) of vascular smooth muscle cells (VSMCs) at the resting state. Effects of (A) T alone and (B) 0.01% ethanol solvent on the $[Ca^{2+}]_i$ of VSMCs at the resting state. VSMCs had good reactivity to high-potassium solution. Arrows indicate corresponding drug stimulations and light-coloured triangles represent the termination of stimulation using HEPES-buffered saline perfusion. The curves shown in the figure are the representative results of 22-25 cells from ≥3 experiments.

To exclude the possible impact of the testosterone solvent (anhydrous ethanol), the effect of 0.01% anhydrous ethanol on the resting state $[Ca^{2+}]_i$ of VSMCs was observed further (Fig. 1B). An increase in $[Ca^{2+}]_i$ was caused by 0.01% anhydrous ethanol and the amplitude was similar to that of testosterone (<50 nM). Thus, the mild elevation of $[Ca^{2+}]_i$ in resting state VSMCs caused by testosterone alone may be interfered by the ethanol solvent. The following Ca^{2+} imaging analyses were performed using 0.01% ethanol as the blank control of testosterone.

Effects of testosterone on the high-potassium solution-induced increase in the $[Ca^{2+}]_i$ of VSMCs. High concentrations of KCl solutions can cause vasoconstriction. Studies have shown that testosterone can produce relaxation responses in arteries precontracted by high-potassium stimulation. Our experiments further observed the effects of testosterone on the high-potassium solution-induced increase in the $[Ca^{2+}]_i$ of VSMCs.

Effects of testosterone intervention on the high-potassium solution-induced increase in the $[Ca^{2+}]_i$ of VSMCs. VSMCs were treated with the high-potassium solution for 30 sec and subsequently reacted with potassium perfusate with 0.01% anhydrous ethanol, with or without 40 nmol/l or 4 μmol/l testosterone for 1 min, followed by observation of $[Ca^{2+}]_i$ changes (Fig. 2A). Stimulation with a high-potassium solution for 30 sec led to a rapid increase in the $[Ca^{2+}]_i$ in VSMCs to the plateau phase level. Compared with the ethanol vehicle, interventions with physiological concentrations of testosterone (40 nmol/l) could significantly inhibit $[Ca^{2+}]_i$ elevation; treatment with supraphysiological concentrations of testosterone (4 μmol/l) also yielded similar effects (Fig. 2B).

Effects of T-BSA intervention on the high-potassium solution-induced increase in the $[Ca^{2+}]_i$ of VSMCs. Testosterone is a steroid hormone that can directly pass through the cell membrane lipid bilayer into the cytoplasm to bind with AR, where it is subsequently transferred to the nucleus to regulate target gene transcription. When testosterone covalently binds with macromolecular proteins (such as BSA), the resulting complex cannot directly pass through the cell membrane into the cytoplasm. The present experiments further examined the effects of T-BSA intervention on the high-potassium solution-induced increase in the $[Ca^{2+}]_i$ of VSMCs. As the molar ratio at which BSA is coupled with testosterone in commercialised T-BSA molecules is 1:23.6, the T-BSA concentrations used in the observation were higher than pure testosterone (Fig. 3). A BSA-blank control had no significant effects on the high-potassium solution-induced increase in the $[Ca^{2+}]_i$. By contrast, a 90-sec action of 400 nmol/l T-BSA could significantly reduce the $[Ca^{2+}]_i$ (Fig. 3A); intervention with 40 μmol/l T-BSA obtained similar results (Fig. 3B). These results indicate that testosterone inhibits $[Ca^{2+}]_i$ increases via its interaction with the cell membranes of VSMCs.

Effects of repetitive testosterone interventions on the high-potassium solution-induced increase in the $[Ca^{2+}]_i$ of VSMCs. To further understand whether the interaction between testosterone and VSMC membranes is reversible, repetitive interventions of physiological levels of testosterone were observed on the high-potassium solution-induced increase in the $[Ca^{2+}]_i$ of VSMCs (Fig. 4). The $[Ca^{2+}]_i$ of VSMCs responded well to high-potassium solution. After perfusion with HBS-blank solution for 5 min, the $[Ca^{2+}]_i$ gradually returned to baseline levels. Subsequently, after 30 sec of high-potassium solution stimulation, 40 nmol/l of testosterone was added for 90 sec, which significantly reduced the $[Ca^{2+}]_i$. After 5 min of transition with HBS, the above steps were repeated, and testosterone significantly inhibited the high-potassium solution-induced $[Ca^{2+}]_i$ increase. However, the amplitude of the increase was slightly lower than that observed with the first exposure. Thus, the good response that VSMCs had to high-potassium stimulation was verified. These results suggest that the interaction between testosterone and VSMC membranes is reversible.

Effects of testosterone pretreatment on the high-potassium solution-induced increase in the $[Ca^{2+}]_i$ of VSMCs. VSMCs were pretreated with physiological concentrations of testosterone for 2 min and were subsequently stimulated with a high-potassium solution containing 40 nmol/l of testosterone for 2 min. Changes in the $[Ca^{2+}]_i$ were observed (Fig. 5). The $[Ca^{2+}]_i$ of VSMCs responded well to high-potassium solution and pretreatment with testosterone alone mildly increased the $[Ca^{2+}]_i$ (the amplitude of the increase was similar to that of the ethanol control). Subsequent treatment with high-potassium

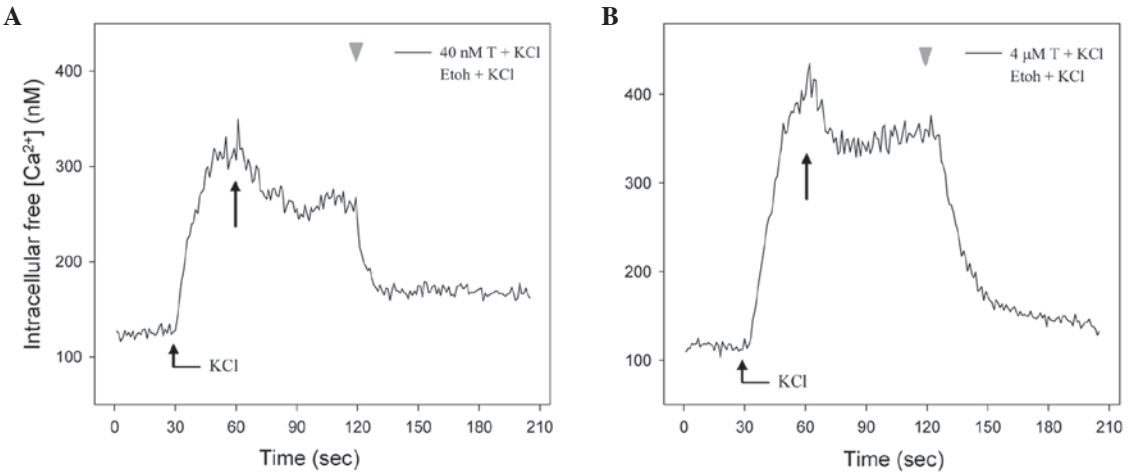

Figure 2. Effects of testosterone (T) intervention on the high-potassium solution-induced increase in the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) of vascular smooth muscle cells (VSMCs). (A) Effects of T intervention at a (A) physiological (40 nmol/l) and (B) supraphysiological concentration (4 μmol/l) on the high-potassium solution-induced increase in the $[Ca^{2+}]_i$ of VSMCs. Arrows indicate corresponding drug stimulations and light-coloured triangles represent the termination of stimulation using HEPES-buffered saline perfusion. The curves shown in the figure are the representative results of 18-33 cells from ≥3 experiments.

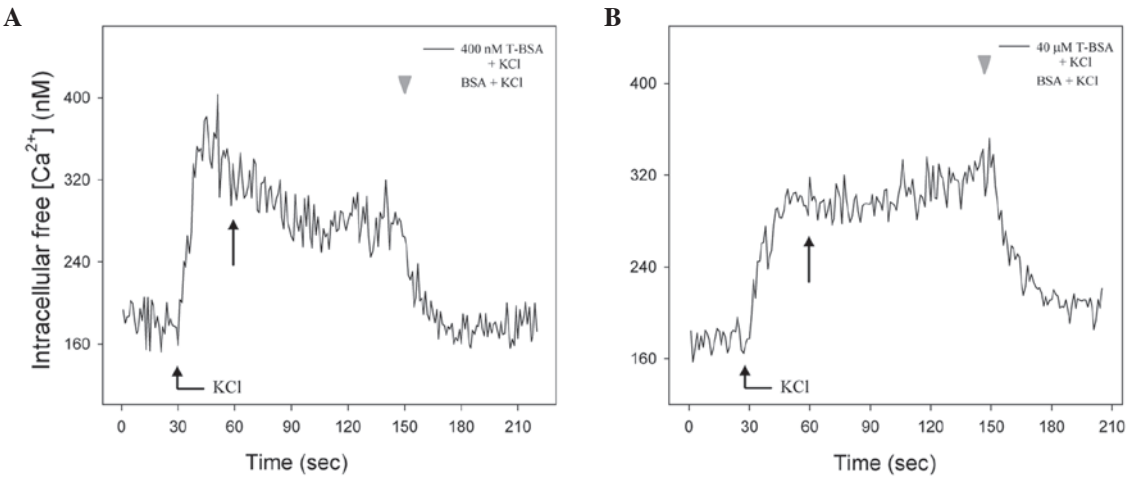

Figure 3. Effects of testosterone coupled to bovine serum albumin (T-BSA) intervention on the high-potassium solution-induced increase in the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) of vascular smooth muscle cells (VSMCs). Effects of (A) T-BSA intervention at 400 nmol/l and (B) 40 μmol/l on the high-potassium solution-induced increase in the $[Ca^{2+}]_i$ of VSMCs. Arrows indicate corresponding drug stimulations and light-coloured triangles represent the termination of stimulation using HEPES-buffered saline perfusion. The curves shown in the figure are the representative results of 19-23 cells from ≥3 experiments.

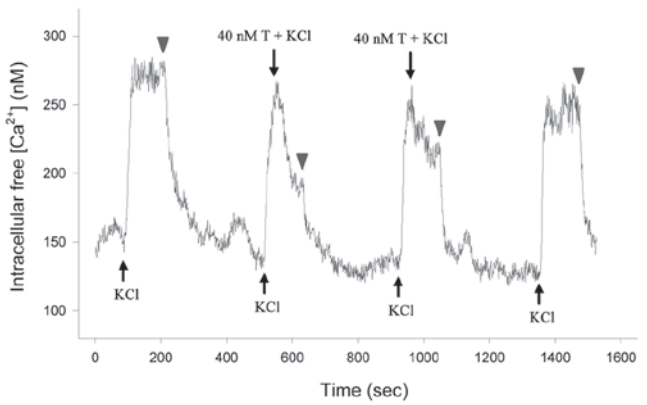

Figure 4. Effects of repetitive testosterone (T) interventions on the high-potassium solution-induced increase in the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) of vascular smooth muscle cells. Arrows indicate corresponding drug stimulations and light-coloured triangles represent the termination of stimulation using HEPES-buffered saline perfusion. The curves shown in the figure are the representative results of 27-30 cells from ≥3 experiments.

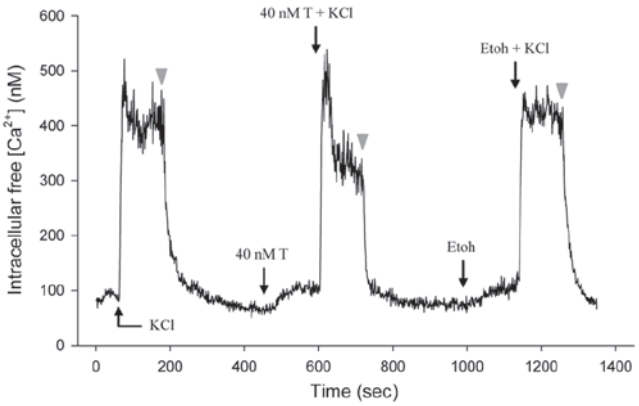

Figure 5. Effects of testosterone (T) pretreatment on the high-potassium solution-induced increase in the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) of vascular smooth muscle cells. Arrows indicate corresponding drug stimulations and light-coloured triangles represent the termination of stimulation using HEPES-buffered saline perfusion. The curves shown in the figure are the representative results of 17-26 cells from ≥3 experiments.

solution containing 40 nmol/l of testosterone for 2 min led to a rapid increase in the $[Ca^{2+}]_i$ to a level similar to that of pure high-potassium stimulation; however, the $[Ca^{2+}]_i$ could not be maintained at that plateau and decreased rapidly. Pretreatment with 0.01% anhydrous ethanol for 2 min had no significant effects on the $[Ca^{2+}]_i$ reaction curve to high-potassium stimulation.

Discussion

In male patients with coronary heart disease, intravenous injection of supraphysiological doses of androgen can significantly delay the time that ST segment depression occurs in exercise tests (11) and direct intracoronary application of physiological concentrations of testosterone can increase coronary blood flow and coronary circumference (12). In addition, testosterone can rapidly induce vasodilation in all arterial vascular beds *in vitro*, including the coronary, mesenteric, iliac, renal and femoral arteries, and the effect is not affected by the removal of endothelium (6), indicating that the vasodilation effect of testosterone is primarily achieved through its direct action on VSMCs. The present study aimed to explore the effects of testosterone on the $[Ca^{2+}]_i$ of VSMCs. To the best of our knowledge, we demonstrated for the first time that testosterone can inhibit $[Ca^{2+}]_i$ increases induced by high concentrations of KCl in primary cultured VSMCs.

Currently, there are two opposing hypotheses for the endothelium-independent mechanism of testosterone-mediated vasodilation, i.e., K^+ channel activation and Ca^{2+} channel antagonism. Using *in vitro* porcine coronary artery as the research subject, Deenadayalu *et al* (13) demonstrated that pretreatment with K_{Ca} channel inhibitors could eliminate the vasodilation effects of testosterone. In addition, applying a patch-clamp technique on the primarily cultured coronary smooth muscle cells, Deenadayalu *et al* further showed that the Ca^{2+}-sensitive maxi-K^+ channel (BK_{Ca}) was the primary channel regulating cellular electrical activity and that testosterone acted by opening the BK_{Ca} channel. By contrast, the research group led by Channer published a series of reports (6,14,15) demonstrating that testosterone has antagonistic effects against Ca^{2+}. They confirmed the relaxation effects of androgen in the coronary and pulmonary arteries of rats and suggested that testosterone had an antagonistic effect on voltage-operated Ca^{2+} channels (VOCCs) and receptor-operated Ca^{2+} channels (ROCCs) (14). Recently, Hall *et al* (15) used A7r5 VSMCs as the research subject and demonstrated that physiological concentrations of testosterone selectively inhibited the L-type Ca^{2+} channels to prevent Ca^{2+} influx. A previous study (16) used whole-cell patch-clamp to confirm that testosterone inhibits Ca^{2+} influx by binding to L-type Ca^{2+} channels. These studies suggest that testosterone-mediated vasodilation relies on specific Ca^{2+}-dependent signalling mechanisms and the majority of studies have supported the conclusion that testosterone has antagonistic effects against Ca^{2+} (14).

High-potassium solution is a common vasocontraction factor. It leads to membrane potential depolarisation by inhibiting K^+ efflux of VSMCs, thereby opening up cell membrane VOCCs, promoting Ca^{2+} influx and causing contraction of VSMCs. The present study confirmed that stimulation of VSMCs using a high-potassium solution could lead to a rapid increase of $[Ca^{2+}]_i$ that was maintained at a high plateau level and that inhibition at the plateau using physiological levels of testosterone (40 nmol/l), along with pretreatment, were able to significantly inhibit the potassium solution-induced $[Ca^{2+}]_i$ increase. These results suggest that testosterone has an inhibitory effect on membrane VOCCs indicating that androgen can not only expand the coronary artery to relieve myocardial ischemia, but can also have a preventive effect on the occurrence of angina pectoris, which is consistent with the results from the research group led by Channer (14). The above clinical trials also confirmed this point. In addition, alternative T-BSA intervention also showed inhibitory effects on the high-potassium-stimulated $[Ca^{2+}]_i$ increase, and repeated testosterone interventions in short time windows led to inhibitory effects. These results suggest that these effects of testosterone are achieved through its interaction with the cell membranes of VSMCs and are reversible, indicating the presence of testosterone-specific membrane receptors on the cell membranes of VSMCs.

As a steroid hormone, the classical pathway of androgen is to bind to AR, which acts as a transcription factor and enters the nucleus to exert transcriptional activation and/or inhibition of target genes. This effect can be inhibited by the AR antagonist flutamide. The genomic effect of androgen takes longer to occur as it consists of a series of steroid-induced events, including mRNA generation and modification, and protein synthesis, translocation, transportation and exertion of function. By contrast, non-genomic effects of steroid hormones have several characteristics (17). First, the effects are fast-acting, initiating within a few seconds or minutes. Second, the effects are not sensitive to inhibitors of transcription and protein synthesis. Third, steroids conjugated with macromolecules (such as BSA) cannot enter the cells but still have similar biological activities. The present study confirmed that the short-term (~1 min) action of testosterone significantly inhibited a KCl-induced increase in $[Ca^{2+}]_i$ and that T-BSA of corresponding concentrations had a similar effect, indicating that testosterone acts via non-transcriptional mechanisms to inhibit $[Ca^{2+}]_i$ increases in VSMCs. Previous studies also confirm that androgen regulates AR expression in VSMCs at the transcriptional and non-transcriptional levels. Additionally, the non-transcriptional mechanism does not involve the cAMP-PKA and PKC signalling pathways and is possibly mediated by the Ca^{2+}-calmodulin kinase pathway (18).

In conclusion, testosterone has a non-transcriptional regulatory effect on the $[Ca^{2+}]_i$ of VSMCs and inhibits VOCC-mediated Ca^{2+} influx by interacting with the cell membranes of VSMCs, which may be one of the mechanisms of testosterone-mediated vasodilation. Further in-depth study of the non-transcriptional pathway of testosterone and the possibility of membrane AR interaction may provide new effective means for the prevention and treatment of coronary heart disease in the future.

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