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Testosterone-augmented contractile responses to α_1- and β_1-adrenoceptor stimulation are associated with increased activities of RyR, SERCA, and NCX in the heart

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Tsang S, Wong SS, Wu S, Kravtsov GM, Wong T. Testosterone-augmented contractile responses to α_1- and β_1-adrenoceptor stimulation are associated with increased activities of RyR, SERCA, and NCX in the heart. *Am J Physiol Cell Physiol* 296: C766–C782, 2009; doi:10.1152/ajpcell.00193.2008.—We hypothesized that testosterone at physiological levels enhances cardiac contractile responses to stimulation of both α_1- and β_1-adrenoceptors by increasing Ca^{2+} release from the sarcoplasmic reticulum (SR) and speedier removal of Ca^{2+} from cytosol via Ca^{2+}-regulatory proteins. We first determined the left ventricular developed pressure, velocity of contraction and relaxation, and heart rate in perfused hearts isolated from control rats, orchiectomized rats, and orchiectomized rats without and with testosterone replacement (200 μg/100 g body wt) in the presence of norepinephrine (10^{-7} M), the α_1-adrenoceptor agonist phenylephrine (10^{-6} M), or the nonselective β-adrenoceptor agonist isoprenaline (10^{-7} M) in the presence of 5×10^{-7} M ICI-118,551, a β_2-adrenoceptor antagonist. Next, we determined the amplitudes of intracellular Ca^{2+} concentration transients induced by electrical stimulation or caffeine, which represent, respectively, Ca^{2+} release via the ryanodine receptor (RyR) or releasable Ca^{2+} in the SR, in ventricular myocytes isolated from the three groups of rats. We also measured $^{45}Ca^{2+}$ release via the RyR. We then determined the time to 50% decay of both transients, which represents, respectively, Ca^{2+} reuptake by sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) and removal via the sarcolemmal Na^+/Ca^{2+} exchanger (NCX). We correlated Ca^{2+} removal from the cytosol with activities of SERCA and its regulator phospholamban as well as NCX. The results showed that testosterone at physiological levels enhanced positive inotropic and lusitropic responses to stimulation of α_1- and β_1-adrenoceptors via the androgen receptor. The increased contractility and speedier relaxation were associated with increased Ca^{2+} release via the RyR and faster Ca^{2+} removal out of the cytosol via SERCA and NCX.

orchiectomy; androgen receptor; left ventricular developed pressure; velocity of contraction and relaxation; electrical-induced intracellular Ca^{2+} concentration transients; caffeine-induced intracellular Ca^{2+} concentration transients; ryanodine receptor; sarco(endo)plasmic reticulum Ca^{2+}-ATPase; Na^+/Ca^{2+} exchanger

NOREPINEPHRINE (NE) released from the nerve terminals of the sympathetic nervous system activates both α_1- and β_1-adrenoceptors (33, 53), which regulates contractile responses in the myocardium. It is well documented that β_1-adrenoceptor activation increases contraction and accelerates relaxation (10, 64). There is also evidence showing that α_1-adrenoceptor activation triggers positive inotropic responses in the rat myocardium (6, 14). We recently demonstrated that testosterone enhances injury responses and contractile recovery to stimulation of both α_1- and β_1-adrenoceptors as well as enhances the expression of both adrenoceptor subtypes (56). In addition, Golden et al. (18) demonstrated that testosterone also increases the expression of L-type Ca^{2+} channels and the Na^+/Ca^{2+} exchanger (NCX), the activities of which are augmented by activation of β_1- and α_1-adrenoceptors, respectively. These observations prompted us to hypothesize that testosterone at physiological levels enhances cardiac contractile responses to stimulation of both α_1- and β_1-adrenoceptors in the myocardium, which is associated with increased Ca^{2+} release and speedier Ca^{2+} removal via Ca^{2+}-regulatory proteins.

The contractile function of the heart is determined by intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) handling, which, in turn, is determined by the expression and activity of Ca^{2+}-regulatory proteins. Depolarization of the sarcolemma opens voltage-gated L-type Ca^{2+} channels, thus increasing the influx of Ca^{2+} into the cytosol. This Ca^{2+} influx induces a massive release of Ca^{2+} from the sarcoplasmic reticulum (SR) via the ryanodine receptor (RyR), resulting in a sudden increase in Ca^{2+} and a $[Ca^{2+}]_i$ transient that triggers contraction. Subsequent to contraction, Ca^{2+} is sequestered to the SR via sarco(endo)plasmic Ca^{2+}-ATPase (SERCA) and out of the cell via NCX. It is well established that SERCA is under the tight control of a closely associated SR membrane protein, phospholamban (PLB), which can be phosphorylated at two adjacent sites, Ser^{16} and Thr^{17} (22). Dephosphorylated PLB inhibits SERCA, whereas phosphorylation of PLB reverses this inhibition. In the rat ventricular myocyte, SERCA and NCX are responsible for >90% and ~7% of Ca^{2+} removal, respectively (4, 28), and are the main mechanisms responsible for relaxation.

The dynamic changes of $[Ca^{2+}]_i$ can be revealed by $[Ca^{2+}]_i$ transients induced by electrical stimulation ($E[Ca^{2+}]_i$ transient) or caffeine administration ($C[Ca^{2+}]_i$ transient). The $E[Ca^{2+}]_i$ transient is triggered by electrical stimulation, which depolarizes the sarcolemma, as does the action potential. The amplitude and time to 50% decay of the transient (T_{50}) values of the $E[Ca^{2+}]_i$ transient, therefore, reveal the Ca^{2+} release into and removal from the cytosol during excitation-contraction coupling, which directly correlate to contraction and relaxation of the cardiac muscle (70). Caffeine opens the RyR, thus depleting Ca^{2+} in the SR. Therefore, the amplitude of the $C[Ca^{2+}]_i$ transient represents the Ca^{2+} in the SR available for release and the T_{50} represents the rate of removal of Ca^{2+} from the

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cytosol. Despite the testosterone-mediated upregulation on L-type Ca^{2+} channels and NCX, Callies et al. (8) reported a lack of effect of testosterone on the basal expression of PLB, RyR type 2, and SERCA 2a (8). Nevertheless, the effect of cross-talk between testosterone and adrenoceptors on Ca^{2+} homeostasis and the activity of Ca^{2+}-regulatory proteins has not been reported.

In an attempt to test the hypothesis that testosterone enhances cardiac contractile responses to adrenoceptor stimulation, which are associated with altered Ca^{2+} handling involving altered activities of Ca^{2+}-regulatory proteins, we first determined the contractile variables, namely, left ventricular (LV) developed pressure (LVDP), velocity of contraction and relaxation ($+dP/dt_{max}$ and $-dP/dt_{max}$, respectively), and heart rate (HR), in response to adrenoceptor activation in perfused hearts isolated from control rats, orchiectomized (ORX) rats, and ORX rats without and with testosterone (200 μg/100 g body wt) replacement (ORX + T). We then studied the Ca^{2+} homeostasis underlying the changes in contractility by examining the amplitudes of $E[Ca^{2+}]_i$ transients and $C[Ca^{2+}]_i$ transients, which represent, respectively, the release of Ca^{2+} via the RyR and releasable Ca^{2+} from the $[Ca^{2+}]_i$ store, the SR. We also measured $^{45}Ca^{2+}$ release via the RyR. We then correlated the Ca^{2+} release from the SR with activities of the RyR. We further determined the Ca^{2+} homeostasis underlying the relaxation by determining the T_{50} of $E[Ca^{2+}]_i$ and $C[Ca^{2+}]_i$ transients, which represent Ca^{2+} reuptake by SERCA and removal via NCX, respectively. We also correlated the Ca^{2+} removal from the cytosol with activities of SERCA and its critical regulator PLB as well as sarcolemmal NCX.

The results showed that *1*) testosterone at physiological levels enhanced positive inotropic and lusitropic responses to stimulation of α_1- and β_1-adrenoceptors via the androgen receptor; *2*) the increased contractility and speedier relaxation were associated with increased Ca^{2+} release from the SR and faster Ca^{2+} removal from the cytosol; and *3*) the increased release of Ca^{2+} from the SR correlated well with testosterone-augmented activity of the RyR, whereas the faster removal from the cytosol correlated well with enhanced activity of sarcolemmal NCX in the case of α_1-adrenoceptors and enhanced SERCA activity, accompanied with increased phosphorylation of PLB at Ser^{16}, in the case of β_1-adrenoceptors.

MATERIALS AND METHODS

Experimental animals. This study was approved by the Committee on the Use of Live Animals in Teaching and Research of the University of Hong Kong. These investigations conformed to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (NIH Pub. No. 85-23, Revised 1996). Adult male Sprague-Dawley rats weighing 300–350 g were purchased from Charles River Breeding Laboratories (Wilmington, MA).

Testosterone replacement. Male Sprague-Dawley rats were randomly divided into two groups. One group was sham operated and served as the control group. The other group underwent bilateral ORX and was divided into two subgroups. One week after ORX, one subgroup was supplemented with a physiological dose of testosterone (200 μg/100 g body wt sc) daily for 8 wk (the ORX + T group), according to a previous study (3), and another subgroup was treated with vehicle. All surgical procedures were performed under anesthesia with pentobarbital sodium (60 mg/kg ip, Abbott Laboratory, Chicago, IL). Preliminary experiments in our laboratory showed that there were no significant differences in the viability of myocytes between the three groups immediately after isolation, indicating that testosterone deficiency as a result of removal of the testes did not affect the viability of cardiac myocytes. In the series of experiments that determined the hemodynamic variables of cardiac contractile responses (Fig. 1) as well as the activity of Ca^{2+}-regulatory proteins, we used perfused hearts isolated from control, ORX, and ORX + T (200 μg/100 g body wt) rats. In the series of experiments that examined the underlying $[Ca^{2+}]_i$ homeostasis and the role of the androgen receptor, testosterone at physiological relevant concentrations (10^{-8} M) (46, 60) was administered to the same batch of myocytes isolated from ORX rats for 24 h (ORX + T; 10^{-8} M). To confirm the effects of testosterone and α_1- or β_1-adrenoceptor stimulation on $[Ca^{2+}]_i$ transients were indeed α_1- or β_1-adrenoceptor mediated, we administered the α_1- or β_1-adrenoceptor antagonist to the ORX + T group, respectively (see Fig. 5, *A* and *C*). In attempt to study the role of the androgen receptor, we examined the effect of androgen receptor blockade with the androgen receptor antagonist cyproterone acetate (Cyp; 10^{-6}M) on $[Ca^{2+}]_i$ transients in the ORX + T group when the α_1- or β_1-adrenoceptor was activated (see Fig. 5, *E* and *F*).

Immunoassays. Nine weeks after ORX, rats were anesthetized with pentobarbital sodium (60 mg/kg ip) before decapitation by guillotine. Blood samples were allowed to clot, and sera were separated by centrifugation and stored at −80°C until assayed. Total serum testosterone was determined by a commercially available RIA (Diagnostic Products, Los Angeles, CA; sensitivity: 0.4 nM) according to the manufacturer's instructions.

Experimental protocol. In the first series of experiments, we determined the cardiac contractile variables (LVDP, $+dP/dt_{max}$, $-dP/$

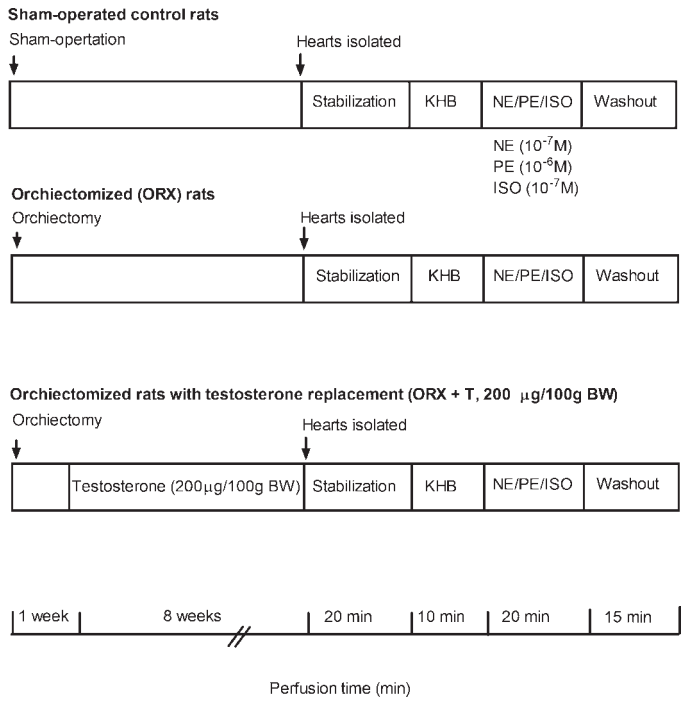


Fig. 1. Experimental protocol to determine the effects of α_1- and/or β_1-adrenoceptor stimulation on hemodynamic variables in perfused hearts isolated from control, orchiectomized (ORX) rats, and ORX rats with testosterone (200 μg/100 g body wt) replacement (ORX + T rats). Hearts isolated from control, ORX, and ORX + T rats were perfused with Krebs-Henseleit buffer (KHB). α_1- and/or β_1-adrenoceptors were stimulated with norepinephrine (NE; 10^{-7} M), the selective α_1-adrenoceptor agonist phenylephrine (PE; 10^{-6} M) in the presence of propranolol (Plol; 10^{-6} M), or the nonselective β-adrenoceptor agonist isoprenaline (Iso; 10^{-7} M) in the presence of the β_2-adrenoceptor antagonist ICI-118,551 (ICI; 5×10^{-7}M) and the α-adrenoceptor antagonist phentolamine (Ptm; 10^{-6} M) for 20 min.

dt_{max}, and HR) in hearts isolated from control, ORX, and ORX + T (200 μg/100 g body wt) rats. Hearts were perfused with Krebs-Henseleit buffer (KHB) for 30 min followed by a 20-min perfusion with the adrenoceptor agonist NE (10^{-7} M), isoprenaline (Iso; 10^{-7} M), or phenylephrine (PE; 10^{-6}M) and a 15-min washout with standard KHB (Fig. 1).

In the second series of experiments, we studied the $[Ca^{2+}]_i$ homeostasis underlying the contractile responses. We first looked at the overall picture of Ca^{2+} handling by measuring both E$[Ca^{2+}]_i$ transients and C$[Ca^{2+}]_i$ transients, which not only provide information on the overall changes in Ca^{2+} homeostasis but also indirectly provide indication of Ca^{2+} handling by the three main Ca^{2+}-handling proteins, namely, the RyR, SERCA, and NCX. A ventricular myocyte was selected after stabilization in the bathing chamber. Changes in $[Ca^{2+}]_i$ were recorded for 10 min, which constituted the basal $[Ca^{2+}]_i$. This was followed by electrical stimulation at 0.2 Hz for the measurement of the E$[Ca^{2+}]_i$ transient (Fig. 2) or direct administration with 10 mM caffeine for the measurement of the C$[Ca^{2+}]_i$ transient (Fig. 3) in presence of the adrenoceptor agonist NE (10^{-7}M), Iso (10^{-7} M), or PE (10^{-6} M) and perfusion for 10 min. In both heart and myocyte preparations, α_1-adrenoceptor stimulation with PE (10^{-6}M) was accompanied with the β-adrenoceptor antagonist propranolol (10^{-6} M) and β_1-adrenoceptor stimulation with Iso (10^{-7} M) was accompanied with the β_2-adrenoceptor antagonist ICI-118,551 (ICI; 5×10^{-7} M) and the α-adrenoceptor antagonist phentolamine (Ptm; 10^{-6} M).

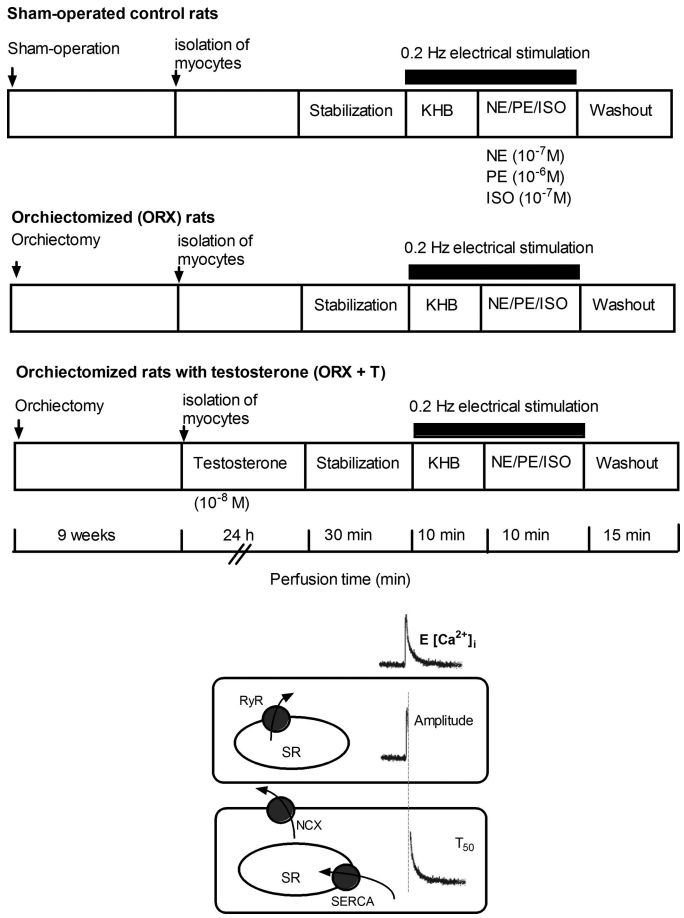


Fig. 2. Experimental protocol to determine the effects of α_1- and/or β_1-adrenoceptor stimulation on intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) transients induced by electrical stimulation (E$[Ca^{2+}]_i$ transients) in ventricular myocytes isolated from control, ORX, and ORX + T rats. Ventricular myocytes were isolated from control, ORX, and ORX + T rats. They were subjected to 0.2 Hz of electrical field stimulation with a 15-ms pulse at 60 V. α_1- and/or β_1-adrenoceptor stimulation was achieved, respectively, by the administration of NE (10^{-7} M), the selective α_1-adrenoceptor agonist PE (10^{-6} M) in the presence of Plol (10^{-6} M), or the nonselective β-adrenoceptor agonist Iso (10^{-7} M) in the presence of the β_2-adrenoceptor antagonist ICI (5×10^{-7} M) and the α-adrenoceptor antagonist Ptm (10^{-6} M) for 10 min. RyR, ryanodine receptor; SR, sarcoplasmic reticulum; NCX, Na^+/Ca^{2+} exchanger; SERCA, sarco(endo)plasmic reticulum Ca^{2+}-ATPase; T_{50}, time to 50% decay of the transient.

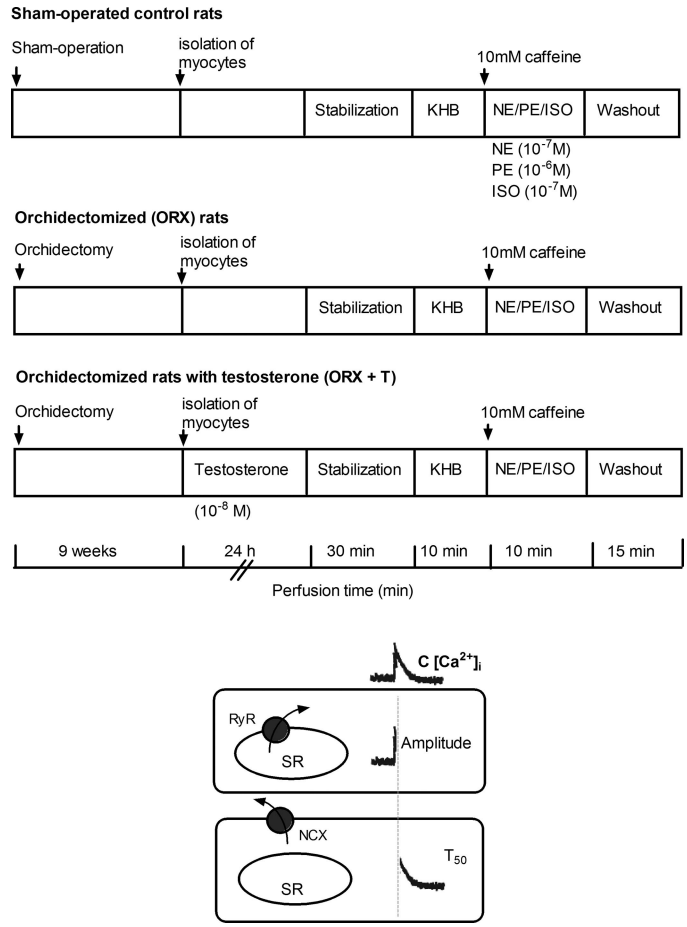


Fig. 3. Experimental protocol to determine the effects of α_1- and/or β_1-adrenoceptor stimulation on $[Ca^{2+}]_i$ transients induced by caffeine (C$[Ca^{2+}]_i$ transients) in ventricular myocytes isolated from control, ORX, and ORX + T rats. Ventricular myocytes were isolated from control, ORX, and ORX + T rats. C$[Ca^{2+}]_i$ transients were recorded by applying 10 mM caffeine directly to the ventricular myocyte. α_1- and/or β_1-adrenoceptors were stimulated, respectively, with the selective α_1-adrenoceptor agonist PE (10^{-6} M) in the presence of Plol (10^{-6} M) or the nonselective β-adrenoceptor agonist Iso (10^{-7} M) in the presence of the β_2-adrenoceptor antagonist ICI (5×10^{-7}M) and the α-adrenoceptor antagonist Ptm (10^{-6} M) for 10 min.

Table 1. *General feature of the experimental animals*

Group	Body Weight, g	Heart Weight, g	Heart Weight/ Body Weight, %	Serum Total Testosterone, ng/ml
Control	538±13.7	2.18±0.05§	0.4±0.011	5.28±0.96
ORX	482±6.65*	1.67±0.04†	0.34±0.011*	<0.4
ORX + T	521±15.2	1.99±0.06‡	0.37±0.018	5.83±1.49

Values are means ± SE; n = 9–10 rats/group. ORX, orchiectomized rats; ORX + T, ORX rats with testosterone (200 μg/100 g body wt) replacement. *$P < 0.05$ vs. the control group; †$P < 0.001$ vs. the control group; ‡$P < 0.05$ vs. the ORX group; §$P < 0.05$ vs. ORX + T group.

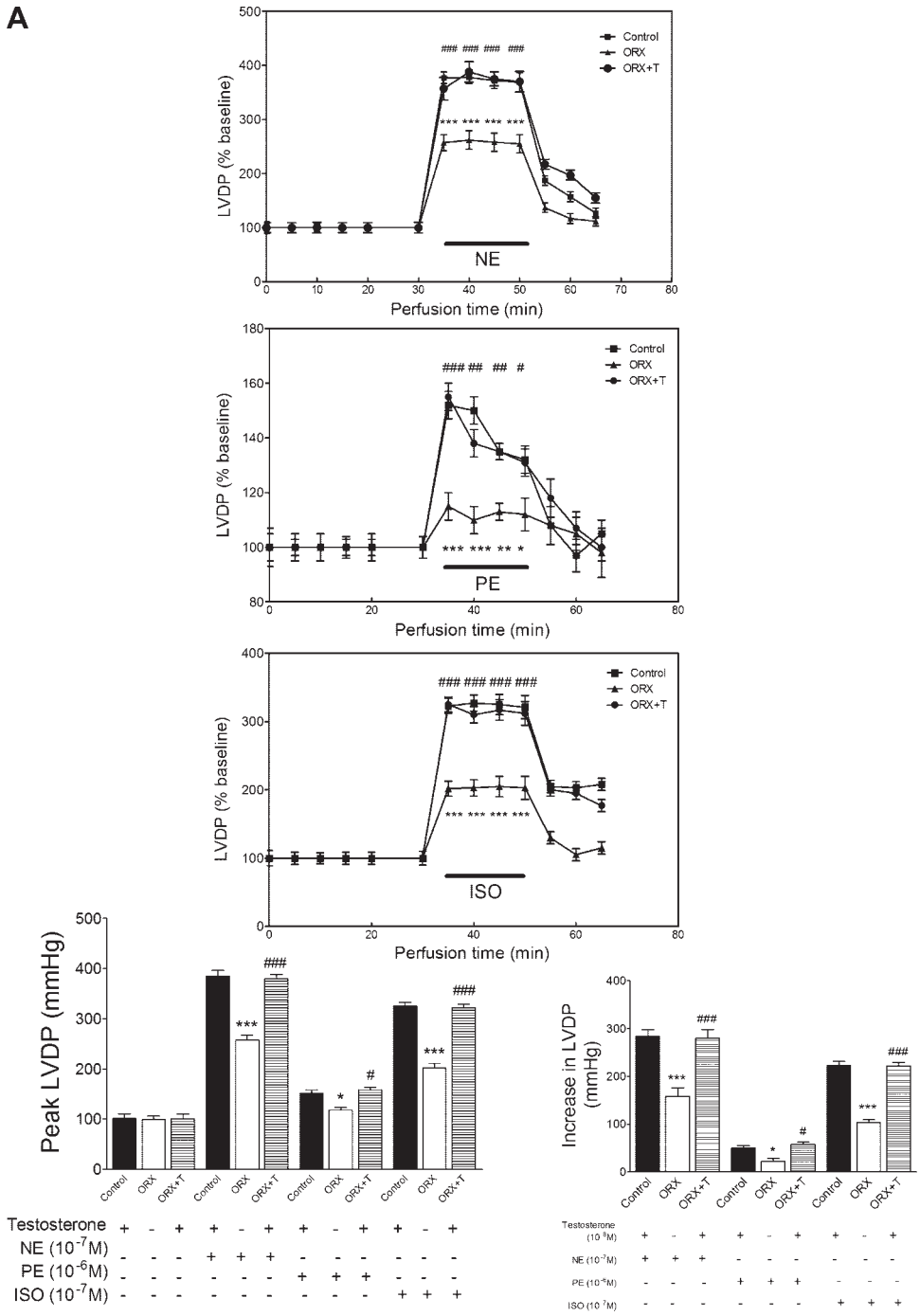


Fig. 4. Effects of α_1- and/or β_1-adrenoceptor stimulation on hemodynamic variables in perfused hearts isolated from control, ORX, and ORX + T rats. *A*: left ventricular developed pressure (LVDP); *B*: velocity of contraction ($+dP/dt_{max}$); *C*: velocity of relaxation ($-dP/dt_{max}$); *D*: heart rate [HR; beats/min (BPM)]. *Top*, time-course changes in hemodynamic variables. Analyses were made during stabilization with KHB (baseline), adrenoceptor agonist dosing, and washout. Data are expressed as percentages of the baseline. Each individual heart received single extended doses (10^{-7} M NE; α_1-adrenoceptor: 10^{-6} M PE; and β_1-adrenoceptor: 10^{-7} M Iso) for 20 min. The NE-induced increase was significantly enhanced in control and ORX + T hearts. *Bottom left*, peak values; *bottom right*, agonist-induced changes in hemodynamic variables. Values are means ± SE of 9–10 hearts from control, ORX, and ORX + T rats. A between-group comparison of the baseline values showed no statistical differences for hemodynamic variables. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ vs. the corresponding control groups; $\#P < 0.05$, $\#\#P < 0.01$, and $\#\#\#P < 0.001$ vs. the corresponding ORX groups.

Perfused isolated heart preparation. Nine weeks after ORX, rats were anesthetized with pentobarbital sodium (60 mg/kg ip) and given heparin (200 IU iv) before decapitation by guillotine. Hearts were excised immediately and placed in ice-cold KHB before being mounted on a Langendorff apparatus for perfusion. Isolated hearts were perfused retrogradely with KHB [which contained (in mM) 118 NaCl, 5 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 1.25 $CaCl_2$, 25 $NaHCO_3$, and 11 glucose] equilibrated with 95% O_2 + 5% CO_2 at a constant pressure of 80 cmH_2O (58) and temperature of 37°C. Transient ventricular fibrillation occurred in all groups. Hearts exhibiting arrhythmias during stabilization were discarded. The cardiac variables of LVDP, $+dP/dt_{max}$, $-dP/dt_{max}$, and HR were monitored continuously by a PowerLab system (ML750 PowerLab/4sp with MLT0380 Reusable BP Transducer, AD Instruments, Colorado Springs, CO). A latex balloon inserted through left atrium into the LV was adjusted to a mean LV end-diastolic pressure to 6–10 mmHg.

Preparation of ventricular myocytes. Ventricular myocytes were isolated from the hearts of control, ORX, and ORX + T rats using the collagenase perfusion method as previously described (59, 62). After isolation, they were allowed to stabilize for at least 30 min before experiments. The yield of myocytes was determined microscopically using a hemocytometer. Myocyte viability was assessed by both Cell Titer Glo reagent (Promega, Madison, WI) and trypan blue exclusion (39, 44, 62). Preparations were considered satisfactory only if rods accounted for >80% of the counted cells at the beginning of each experiment. Myocytes were then plated in minimal essential medium

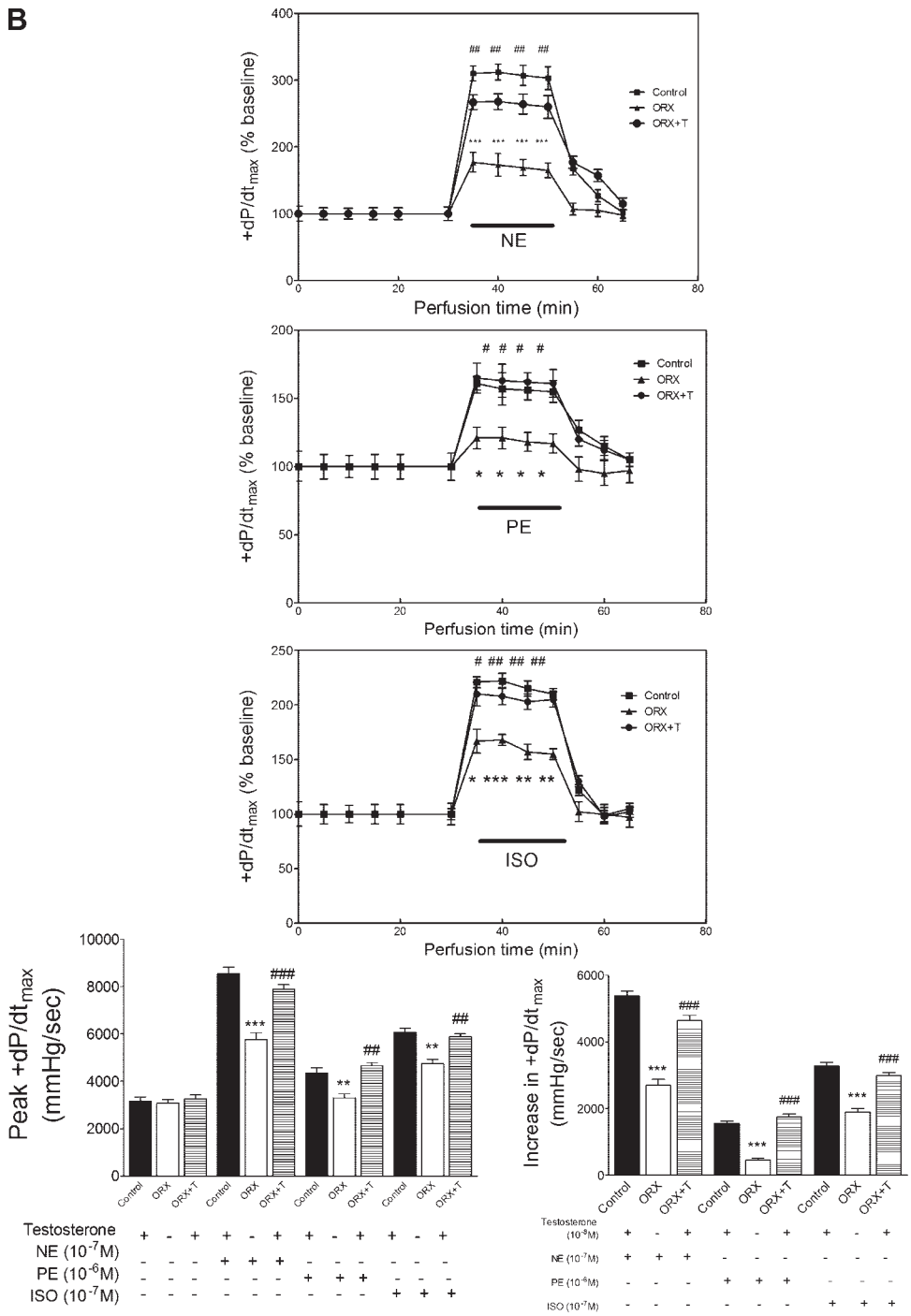


Fig. 4—*Continued*

(MEM) containing 1.25 mM Ca^{2+}, 5% FBS, 5 μM insulin, 5 μM apo-transferrin, 100 U/ml penicillin G, and 100 μM streptomycin on laminin-coated (2 $\mu g/cm^2$, Sigma, St. Louis, MO) plates for 1 h. The plating medium was changed to serum-free medium, and myocytes were incubated at 37°C in 5% CO_2.

Measurement of $[Ca^{2+}]_i$. A spectrofluorometric method with fura-2 AM as the Ca^{2+} indicator was loaded in cells. Fluorescence signals obtained at excitation wavelengths of 340 nm (F_{340}) and 380 nm (F_{380}) were recorded as previously described (62). The F_{340}-to-F_{380} ratio (F_{340}/F_{380} ratio) represented $[Ca^{2+}]_I$ in the myocyte. Ventricular myocytes in all groups selected for the study were rod shaped and quiescent with clear striations. They exhibited asynchronous contraction in response to 0.2 Hz of electrical field stimulation with a 15-ms pulse at 60 V through two platinum wires in the bathing chamber. Myocytes were superfused with KHB that contained (in mmol/l) 118 NaCl, 5 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 1 $CaCl_2$, 25 $NaHCO_3$, and 11 glucose gassed with 95% O_2 + 5% CO_2 at pH 7.4 throughout the study. The amplitudes of both $E[Ca^{2+}]_i$ and $C[Ca^{2+}]_i$ transients were determined as the difference between the resting and peak $[Ca^{2+}]_i$ levels, whereas T_{50} was used to represent the decay of both transients. In the present study, the amplitude and decay of both $C[Ca^{2+}]_i$ and $E[Ca^{2+}]_i$ transients were measured. The rising phase of the $[Ca^{2+}]_i$ transient is due to Ca^{2+} release through the RyR of the SR by a Ca^{2+}-induced Ca^{2+} release mechanism triggered by Ca^{2+} influx

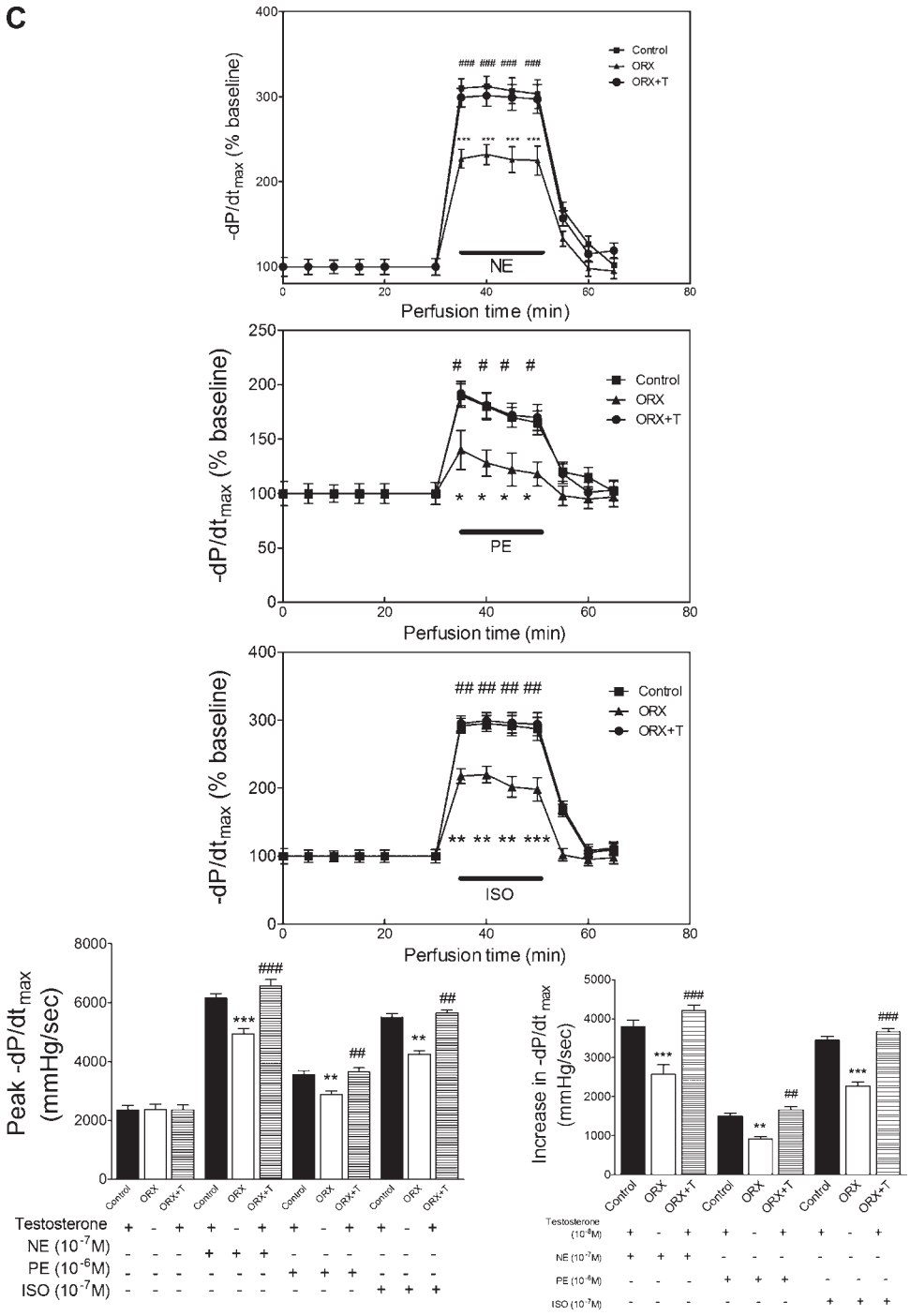


Fig. 4—*Continued*

via the L-type Ca^{2+} channel upon the arrival of the action potential, which, in turn, triggers contraction. The decay phase of the $[Ca^{2+}]_i$ transient is due to sequestration of >90% Ca^{2+} to the SR through SERCA and <10% of Ca^{2+} extruded from the cytosol by sarcolemmal NCX. The $E[Ca^{2+}]_i$ transient, which represents the influx of Ca^{2+} via Ca^{2+} channels and release of Ca^{2+} from the SR triggered by Ca^{2+} influx, directly correlates with cardiac contraction under the present experimental conditions (45, 70). The decay rate of the $E[Ca^{2+}]_i$ transient indicates, mainly, the reuptake of Ca^{2+} by SERCA and removal by sarcolemmal NCX (1, 26, 36). The amplitude of the $C[Ca^{2+}]_i$ transient is an index of the Ca^{2+} content in the SR because caffeine depletes SR Ca^{2+} (26, 29, 54). The decay rate of the $C[Ca^{2+}]_i$ transient reflects Ca^{2+} efflux through sarcolemmal NCX as caffeine keeps the RyR opened (26, 47, 52, 54).

Isolation of the SR and measurement of $^{45}Ca^{2+}$ uptake. SR vesicles were obtained by a previously described method (44, 68). Briefly, freshly isolated cardiac myocytes from rats were homogenized in extraction medium containing (in mM) 40 imidazole HCl, 10 $NaHCO_3$, 5 NaN_3, 250 sucrose, and 1 EDTA (to 2°C, pH 7.0, 5 ml/g tissue) with a Polytron PT 35 homogenizer (Brinkmann, Westbury, NY) at *setting 9* for 10 s each. The homogenate was centrifuged for 5 min at 3,000 *g* to remove cellular debris and further centrifuged at 48,000 *g* for 75 min (Sorvall SM-24 rotor). The supernatant was discarded, and the pellet was suspended in 0.6

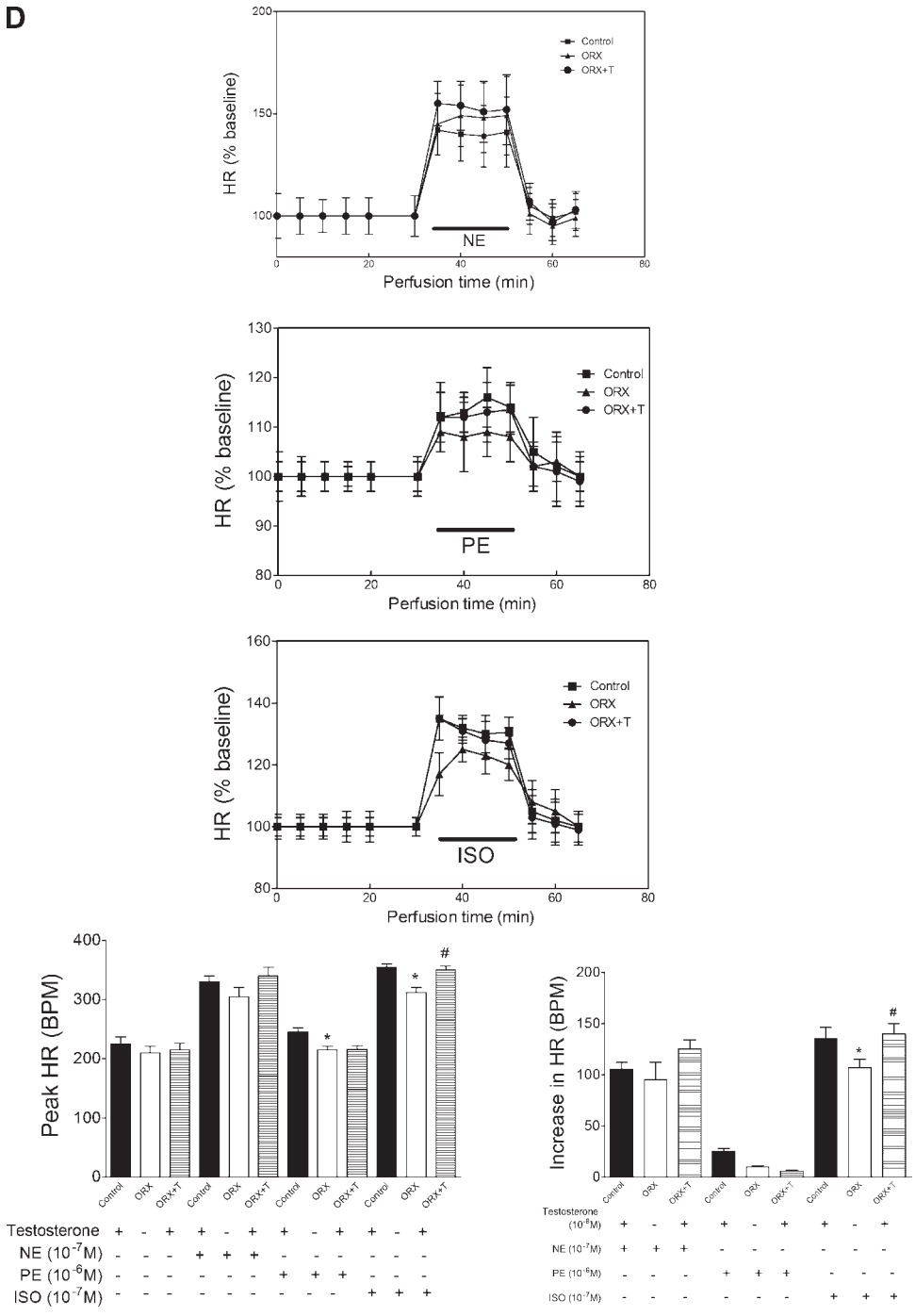


Fig. 4—*Continued*

mM KCl and 20 mM imidazole HCl (pH 7.0) and centrifuged at 48,000 *g* for 60 min (Sorvall SM-24 rotor). The final pellet was rehomogenized in 1 ml of 250 mM sucrose and 40 mM imidazole HCl using a Potter-Elvehjem homogenizer with a Teflon pestle and stored at −70°C. All solutions contained three protease inhibitors, including soybean trypsin inhibitor (40 μg/ml), 0.1% PMSF, and leupeptin (0.5 μg/ml).

The ATP-dependent transport of Ca^{2+} to the SR was measured at room temperature (22°C) using previously described methods (31, 68). SR protein (50–100 μg) was added to 1 ml medium with 40 mM imidazole HCl (pH 7.0), 100 mM KCl, 20 mM NaCl, 5 mM $MgCl_2$, 4 mM ATP-Na_2, 5 K-oxalate, 3 μCi $^{45}CaCl_2$, 5 μM Ru-360 [an inhibitor of Ca^{2+} uptake in mitochondria (69)], and 5 μM calmidazolium [an inhibitor of Ca^{2+}-ATPase of the sarcolemma (42)]. Free [Ca^{2+}] in this solution (5 μM) was determined by Ca^{2+}-EGTA buffer and calculated according to Fabiato and Fabiato (16). After 10 min, aliquots of 0.9 ml were filtered (0.45 μm, Millipore, Bedford, MA). Filters were washed three times with 4 ml of cold (2–4°C) solution containing 40 mM imidazole HCl (pH 7.0), 100 mM KCl, and 0.1 mM EGTA. Subsequent to being washed, filters were placed in vials with 10 ml of scintillation cocktail (Universal LSC cocktail for aqueous samples, Sigma) for ~40 min. The radioactivity was then counted in a scintillation counter (LS 6500, Beckman). The $^{45}Ca^{2+}$ uptake by SERCA, which represents the activity of SERCA, was defined as the difference between the rate of $^{45}Ca^{2+}$ uptake in K-oxalate-containing solution in the presence and absence of 10 μM cyclo-

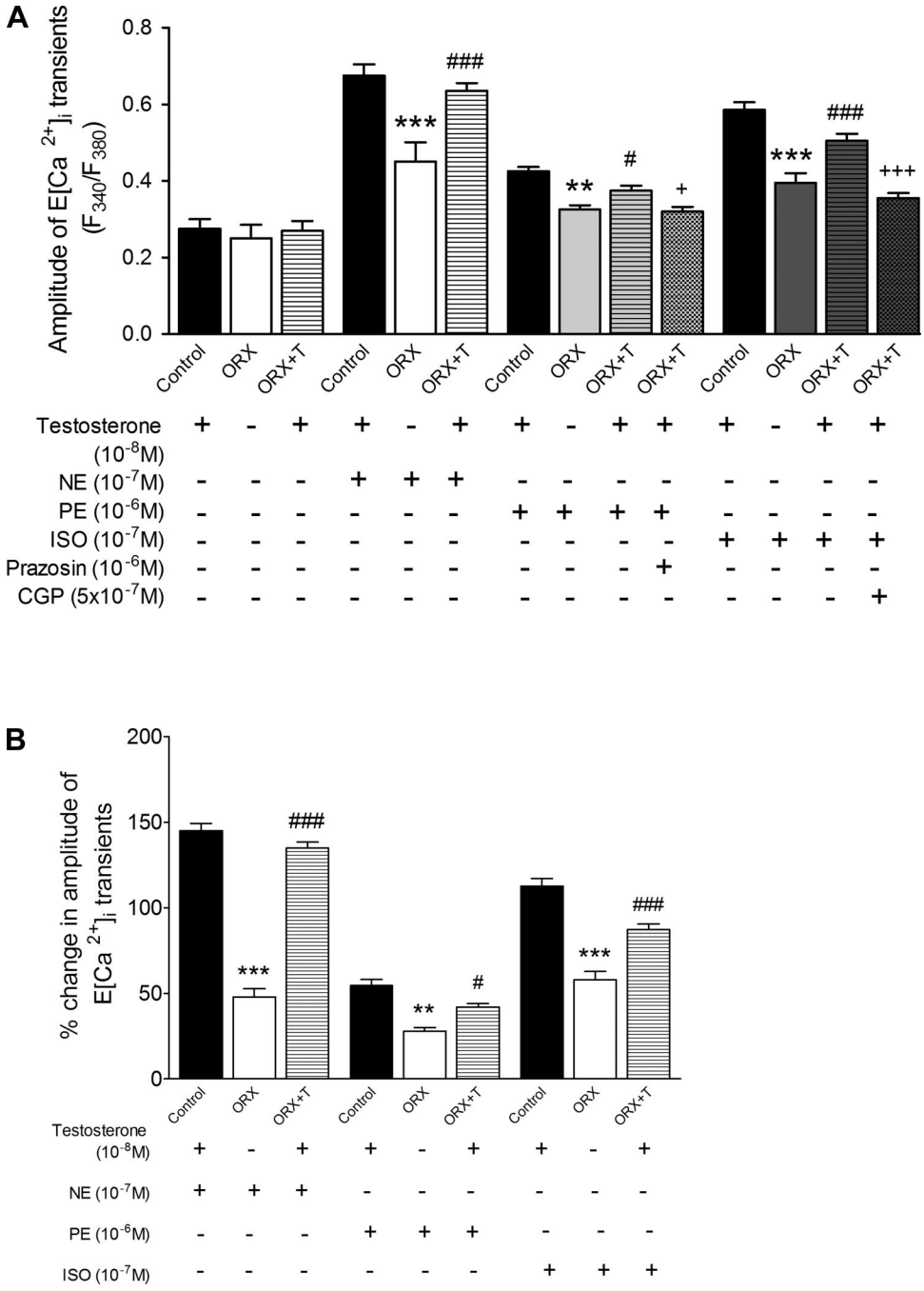


Fig. 5. Effects of α_1- and/or β_1-adrenoceptor stimulation on amplitude (*A* and *E*), T_{50} (*B* and *F*), and percent changes in the amplitude and T_{50} (*C* and *D*) of $E[Ca^{2+}]_i$ transients in ventricular myocytes isolated from control, ORX, and ORX + T rats. α_1- and/or β_1-adrenoceptor stimulation was achieved, respectively, by administration of NE (10^{-7}M), the selective α_1-adrenoceptor agonist PE (10^{-6} M) in the presence of Plol (10^{-6} M), or the nonselective β-adrenoceptor agonist Iso (10^{-7} M) in the presence of the β_2-adrenoceptor antagonist ICI (5×10^{-7} M) and the α-adrenoceptor antagonist Ptm (10^{-6} M) for 10 min. F_{340}/F_{380}, ratio of fluorescence at excitation wavelengths of 340 nm (F_{340}) and 380 nm (F_{380}); CGP, CGP-20712A. Values are means ± SE of 10–15 myocytes from 6 rats/group. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs. the corresponding control groups; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ vs. the corresponding ORX groups; and $^{+}P < 0.05$, $^{++}P < 0.01$, and $^{+++}P < 0.001$ vs. ORX + T rats with Iso/PE.

piazonic acid, a specific inhibitor of SERCA (48). The difference in uptake in the presence and absence of 50 μM ryanodine, a specific blocker of the RyR, was defined as the $^{45}Ca^{2+}$ release via the RyR.

Purification of the plasma membrane and NCX assay. Plasma membrane vesicles were purified as previosuly described (44, 68). Myocytes were sonicated with three 15-s bursts in ice-cold lysis buffer (0.6 M sucrose and 10 mM imidazole HCl, pH 7.0). The homogenate was centrifuged at 1,000 *g* for 5 min to remove cellular debris. The supernatant was centrifuged at 12,000 *g* for 30 min. The supernatant from the 12,000-*g* centrifugation was then diluted in the solution containing 160 mM NaCl, 20 mM HEPES-Tris, and 0.25 M sucrose at pH 7.4 and then further centrifuged at 160,000 *g* for 70 min (Beckman, L8-M, rotor Ti 50.4). The final pellet, which represents the sarcolemma-enriched fraction, was dissolved in 0.5 ml of *solution A*, which contained (in mM) 100 NaCl, 50 LiCl, 6 KCl, and 20 HEPES-Tris (pH 7.4), and then assayed for NCX activity. All solutions contained all three protease inhibitors: 40 mg/ml soybean trypsin inhibitor, 0.1% PMSF, and 0.5 μg/ml leupeptin. Na^+/Ca^{2+} exchange was estimated as specific Na^+-dependent Ca^{2+} uptake according to the previously described protocol (23, 44, 68). Briefly, 4 μl of the vesicle suspension were incubated for 50 min at 22°C to load Na^+ via passive diffusion from the suspension medium, i.e., *solution A*. Subsequently, 15 μl of the vesicle suspension were placed into a tube with 85 μl of K-reaction medium (which contained 160 mM KCl, 0.1 mM $CaCl_2$, 100 μCi $^{45}CaCl_2$, 0.2 mM EGTA, 2 μM valinomycin, 2 μM Ru-360, and 20 mM HEPES-Tris; pH 7.4) to prevent mitochondrial Na^+/Ca^{2+} exchange. The free $[Ca^{2+}]$ in the medium was 55 μM

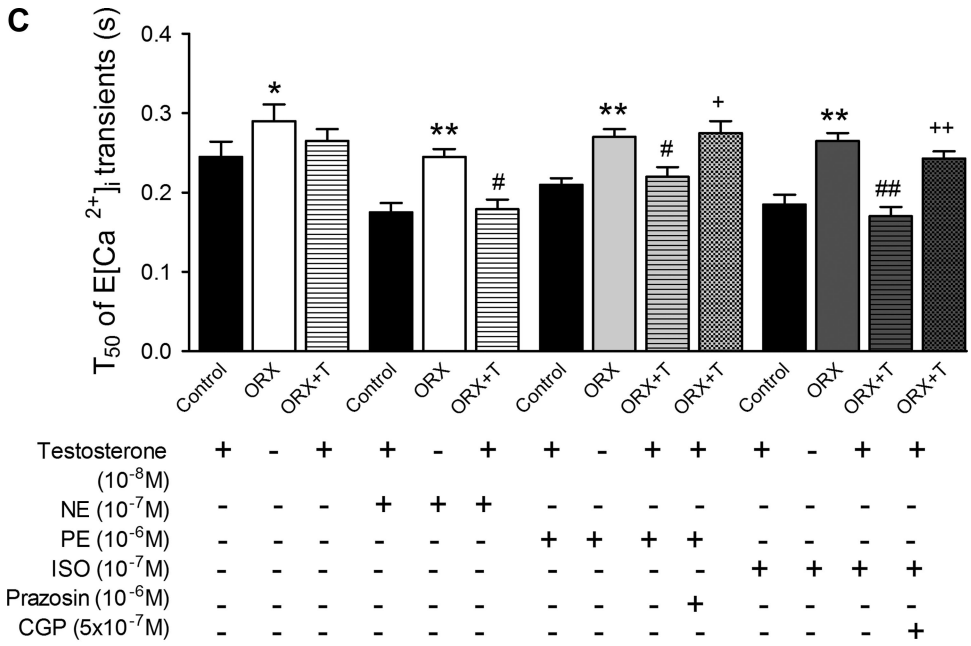
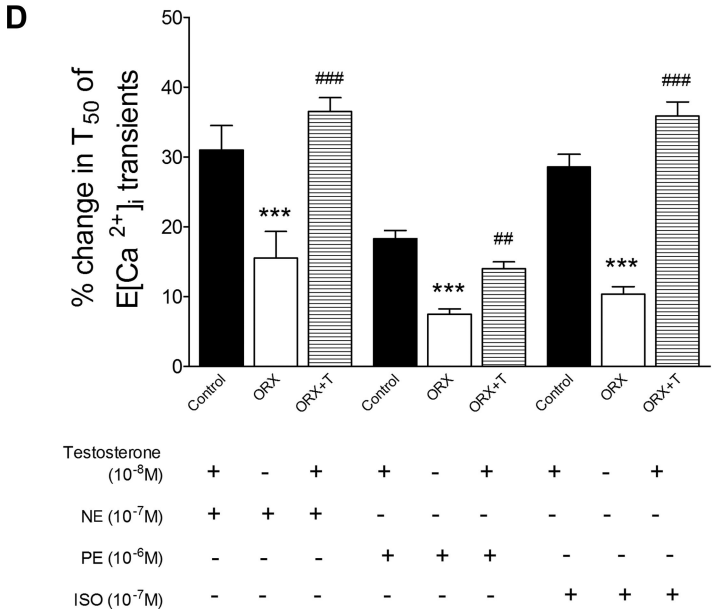


Fig. 5—*Continued*

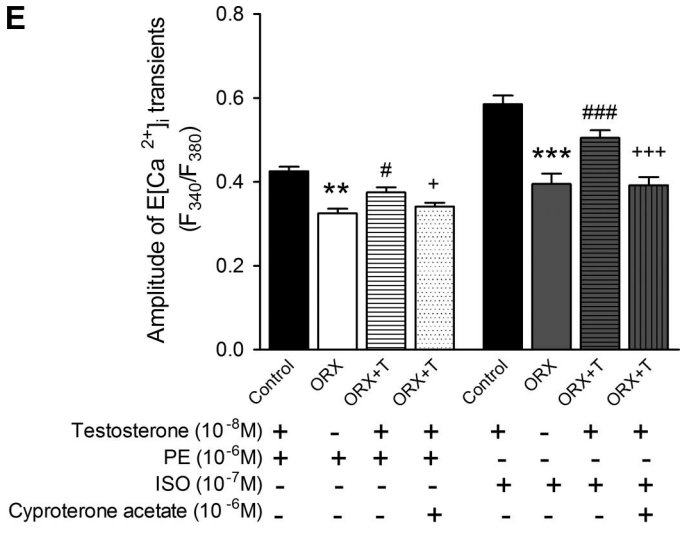


according to the EqCal (Windows Biosoft, 1996) computer program for Ca^{2+}-EGTA buffer. The Ca^{2+} influx was stopped by diluting the reaction mixture at 2, 5, or 10 s with 5 ml of ice-cold termination medium (160 mM KCl and 2 mM $LaCl_3$). Na^+-dependent specific Ca^{2+} uptake was defined as the total Ca^{2+} uptake minus unspecific Ca^{2+} uptake in *solution B* (which contained 0.2 mM EGTA, 0.1 mM $CaCl_2$, 10 μCi $^{45}CaCl_2$, and 2 μM valinomycin), i.e., in a solution where no Na^+ gradient exists across the membrane. All samples were filtered under vacuum, and filters (Millipore, 0.45 μm) were washed twice with 6 ml of 140 mM KCl and 0.1 mM $LaCl_3$. The radioactivity was then counted, and the protein content of each sample was determined using a Bio-Rad assay kit with BSA as the standard.

Western blot analysis. For the immunological detection of PLB phosphorylation sites, 15 μg of SR membrane protein were electrophoresed by SDS-PAGE per lane. Proteins were transferred to polyvinylidene difluoride membranes (Millipore) and probed with polyclonal antibodies raised to PLB (1:200) and PLB (1:200) phosphorylated at Ser^{16} (1:200) or Thr^{17} (1:200) (Santa Cruz Biotechnology). Immunoreactivity was visualized by an ECL detection kit (Amersham Pharmacia Biotech).

Drugs and chemicals. All chemicals were purchased from Sigma unless otherwise stated. Drugs were dissolved in double-distilled H_2O or KHB unless otherwise stated. The stock solution of propranolol was dissolved in ethanol. Testosterone was dissolved in ethanol-DMSO. The final concentration of methanol to ethanol was <0.1% (vol/vol).

Statistical analysis. Data are expressed as means ± SE. Inotropic and chronotropic parameters were analyzed for dose (NE/PE/Iso) and groups (control, ORX, and ORX + T) using repeated-measures ANOVA. Between-group comparisons were performed using ANOVA. The nonparametric Kruskal-Wallis test was used to analyze drug effects. A difference of $P < 0.05$ was considered statistically significant.



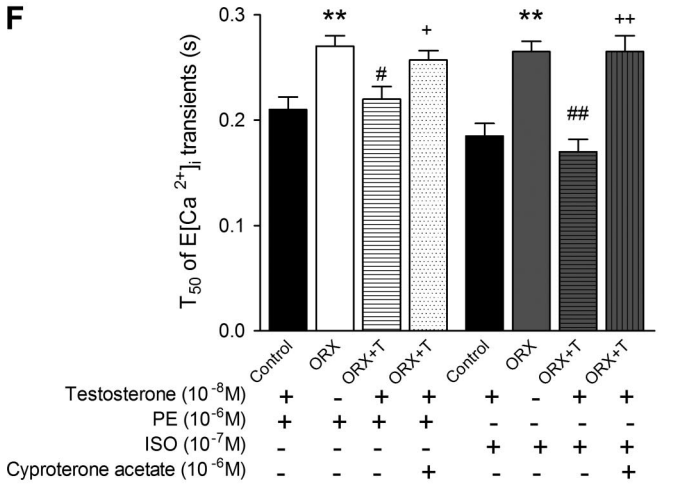


Fig. 5—*Continued*

RESULTS

General features of experimental animals. The serum total testosterone level in the sham-operated control rats was 5.28 ng/ml (18.3 nmol/l). Nine weeks after ORX, the level fell below the detection limit of 0.4 ng/ml (Table 1). Daily injections of testosterone (200 μg/100 g body wt sc) for 8 wk to ORX rats (ORX + T rats) restored the testosterone level to that of control rats. The decrease in the testosterone level in ORX rats was accompanied with significant reductions in body and heart weights and heart weight-to-body weight ratios. With the exception of heart weight, which was restored to normal, these parameters were only partially restored toward normal by testosterone replacement (ORX + T group).

Effects of α_1- and/or β_1-adrenoceptor stimulation on hemodynamic variables in perfused hearts isolated from control, ORX, and ORX + T rats. We first compared hemodynamic variables in perfused hearts isolated from control, ORX, and ORX + T (200 μg/100 g body wt) rats subjected to stimulation with NE (10^{-7} M; which activates both α_1- and β_1-adrenoceptors), the selective α_1-adrenoceptor agonist PE (10^{-6} M) in the presence of the β-adrenoceptor antagonist propranolol (10^{-6} M), or the nonselective β-adrenoceptor agonist Iso (10^{-7} M) in the presence of the β_2-adrenoceptor antagonist ICI (5×10^{-7}M) and the α-adrenoceptor antagonist Ptm (10^{-6} M). There were no statistical differences in baseline values of hemodynamic variables among the three groups in the absence of NE (Fig. 4). Upon the administration of NE, each group showed a significant increase in inotropic and chronotropic responses (Fig. 4). In all groups, an abrupt increase in LVDP occurred over a period of ~1–3 min to a maximum. As shown in the time-course traces (Fig. 4), LVDP, $+dP/dt_{max}$, $-dP/dt_{max}$, and HR remained at constant levels during sustained dosing. Peak values of LVDP, $+dP/dt_{max}$, and $-dP/dt_{max}$ in response to NE were significantly greater in hearts from the control and ORX + T groups than the ORX group (Fig. 4). The administration of either PE (10^{-6} M) or Iso (10^{-7} M) significantly increased LVDP (Fig. 4*A*), $+dP/dt_{max}$ and $-dP/dt_{max}$ (Fig. 4*B*), and HR (Fig. 4*C*) in all groups. With the exception of HR (Fig. 4*D*), the increase was greater in the control group than the ORX group (Fig. 4). Testosterone replacement restored the change in the ORX group to that of the control group (Fig. 4). The increases in LVDP, $+dP/dt_{max}$, and $-dP/dt_{max}$ to peak values in response to NE or PE or Iso were also significantly greater in the control group than the ORX group (Fig. 4, *A–C*). Testosterone replacement restored the increases in LVDP, $+dP/dt_{max}$, and $-dP/dt_{max}$, but not HR, to normal (Fig. 4). The increases in hemodynamic variables in response to NE or PE or Iso were abolished by blockade of the respective receptor subtypes with the selective antagonists prazosin (10^{-6} M) and CGP-20712A (5×10^{-7} M) (data not shown).

Effects of α_1- and/or β_1-adrenoceptor stimulation on the amplitude and T_{50} of the $E[Ca^{2+}]_i$ transient in ventricular myocytes isolated from control and ORX rats with or without testosterone replacement. The basal fluorescence ratio of F_{340}/F_{380}, which indicates the $[Ca^{2+}]_i$ level, was not significantly different among control, ORX, and ORX + T groups (data not shown). In the absence of NE, the amplitude of the $E[Ca^{2+}]_i$ transient was not significantly different among the three groups (Fig. 5*A*). NE (10^{-7} M) or PE (10^{-6} M) or Iso (10^{-7} M) markedly increased the amplitude (Fig. 5*A*) in all three groups. The percent change of the amplitude in the control groups was significantly greater than the corresponding ORX groups (Fig. 5*B*). In the absence of NE, the basal T_{50} of the $E[Ca^{2+}]_i$ transient was significantly longer in the ORX group (Fig. 5*C*). NE (10^{-7} M) or Iso (10^{-7} M) shortened the T_{50} of the $E[Ca^{2+}]_i$ transient in all groups (Fig. 5*C*). PE (10^{-6}M) only shortened the T_{50} in the control and ORX + T groups but not in the ORX group (Fig. 5*C*). The percent change in T_{50} shortening in the control group was significantly greater than the corresponding ORX groups (Fig. 5*D*). The increase in amplitude and shortening of T_{50} were restored by testosterone replacement (Fig. 5, *B* and *D*). Administration of androgen receptor antagonist Cyp (10^{-6} M) abolished the effect of testosterone replacement on both amplitude and T_{50} (Fig. 5, *E* and *F*). The increase in the amplitude and shortening of T_{50} to stimulation of α_1- or β_1-adrenoceptors were also abolished by blockade with the respective selective antagonists prazosin (10^{-6} M) or CGP-20712A (5×10^{-7} M) (Fig. 5, *A* and *C*).

Effects of α_1- and/or β_1-adrenoceptor stimulation on the amplitude and T_{50} of the C[Ca^{2+}]$_i$ transient in ventricular myocytes isolated from control and ORX rats with or without testosterone replacement. The activation of α_1- or β_1-adrenoceptors did not cause marked differences in the amplitude of the C[Ca^{2+}]$_i$ transient among the three groups (Fig. 6*A*). The amplitude of the C[Ca^{2+}]$_i$ transient was slightly lower in ventricular myocytes from ORX rats (Fig. 6*A*). Testosterone replacement for 24 h did not significantly restore the amplitude (Fig. 6*A*). There were no differences in the T_{50} of the C[Ca^{2+}]$_i$ transient among the three groups without adrenoceptor stimulation (Fig. 6*B*). Stimulation of α_1- and β_1-adrenoceptors with PE (10^{-6} M) or Iso (10^{-7} M) significantly decreased the T_{50} of the C[Ca^{2+}]$_i$ transient in both the control and ORX + T groups (Fig. 6*B*). The PE (10^{-6} M)-induced or Iso (10^{-7} M)-induced shortening of T_{50} was significantly greater in the control group than in the ORX group, which was reverted by testosterone replacement (Fig. 6*D*).

Effects of α_1- or β_1-adrenoceptor stimulation on $^{45}Ca^{2+}$ efflux via the SR RyR in ventricular myocytes isolated from control, ORX, and ORX + T rats. We examined ryanodine-sensitive $^{45}Ca^{2+}$ efflux in ventricular myocytes isolated from

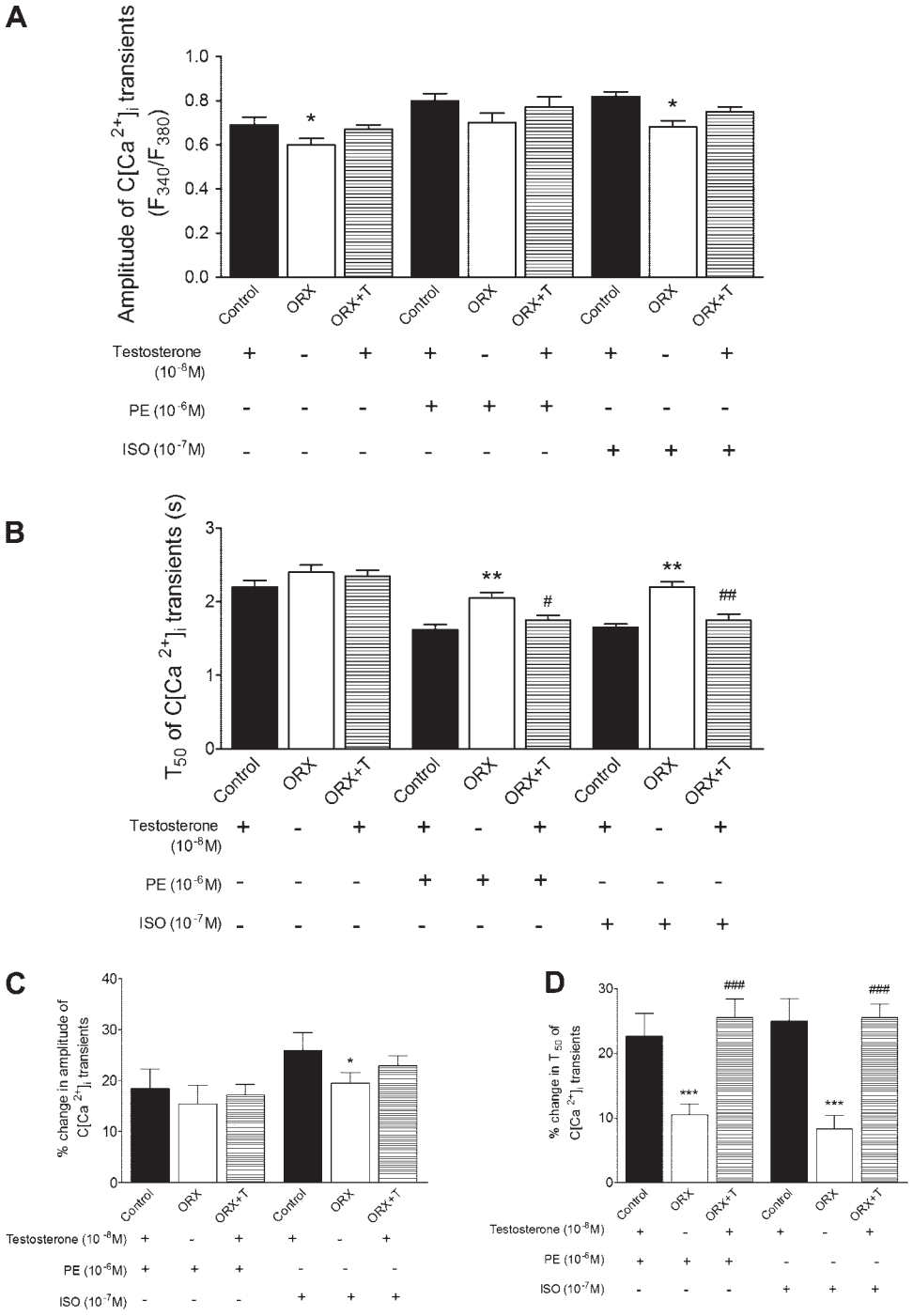


Fig. 6. Effects of α_1- and/or β_1-adrenoceptor stimulation on amplitude (*A*), T_{50} (*B*), and percent changes in amplitude and T_{50} (*C* and *D*) of C[Ca^{2+}]i transients in ventricular myocytes isolated from control, ORX, and ORX + T rats. C[Ca^{2+}]$_i$ transients were recorded by applying 10 mM caffeine directly to the ventricular myocyte. α_1- and/or β_1-adrenoceptors were stimulated with the selective α_1-adrenoceptor agonist PE (10^{-6} M) in the presence of Plol (10^{-6} M) or the nonselective β-adrenoceptor agonist Iso (10^{-7} M) in the presence of the β_2-adrenoceptor antagonist ICI (5×10^{-7} M) and the α-adrenoceptor antagonist Ptm (10^{-6} M) for 10 min. Values are means ± SE of 10–15 myocytes from 6 rats/group. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs. the corresponding control groups; $\#P < 0.05$, $\#\#P < 0.01$, and $\#\#\#P < 0.001$ vs. the corresponding ORX groups.

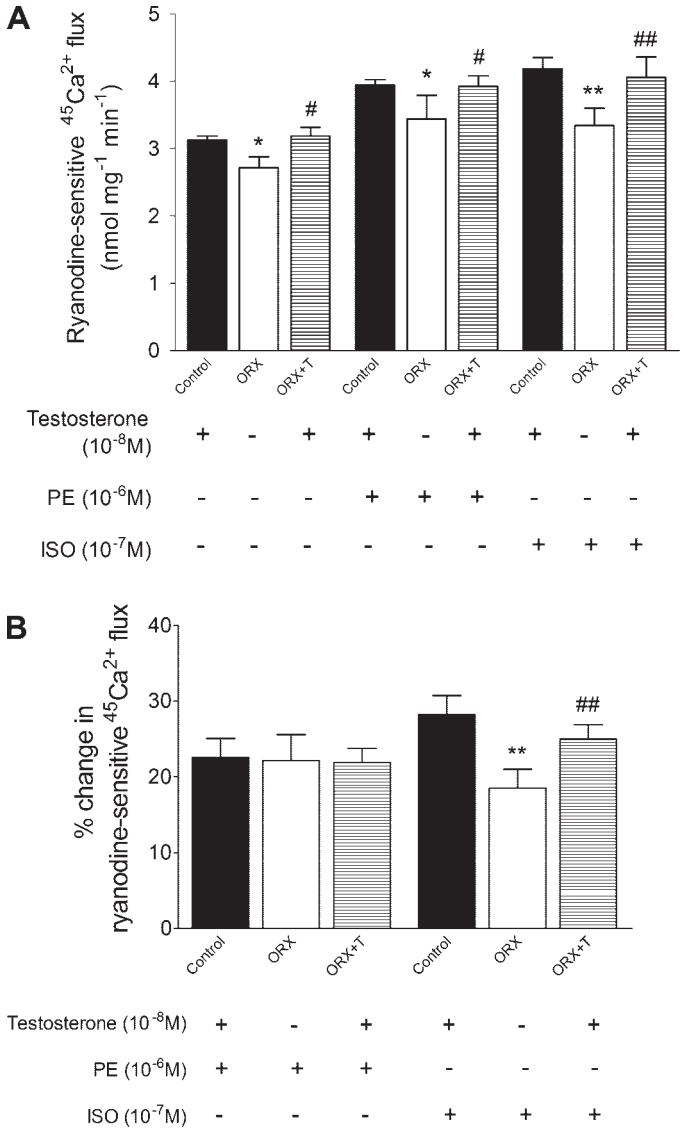


Fig. 7. Effects of α_1- and/or β_1-adrenoceptor stimulation on $^{45}Ca^{2+}$ efflux via the RyR (*A*) and the percent change in $^{45}Ca^{2+}$ efflux via the RyR (*B*) in ventricular myocytes isolated from control, ORX, and ORX + T rats. α_1- and β_1-adrenoceptors were stimulated with the selective α_1-adrenoceptor agonist PE (10^{-6} M) in the presence of Plol (10^{-6} M) or the nonselective β-adrenoceptor agonist Iso (10^{-7} M) in the presence of the β_2-adrenoceptor antagonist ICI (5×10^{-7}M) and the α-adrenoceptor antagonist Ptm (10^{-6} M). Values are means ± SE of 5–6 rats/group. $^*P < 0.05$ and $^{**}P < 0.01$ vs. the corresponding control groups; $\#P < 0.05$ and $\#\#P < 0.01$ vs. the corresponding ORX groups.

control, ORX, and ORX + T rats. In the absence of adrenoceptor stimulation, the basal RyR activity was lower in the ORX group than the control group (Fig. 7*A*). PE (10^{-6} M) or Iso (10^{-7} M) increased $^{45}Ca^{2+}$ fluxes via the RyR in all groups (Fig. 7*A*). The percent change in $^{45}Ca^{2+}$ efflux was significantly greater in the control group than in the ORX group only upon administration of Iso (10^{-7} M) but not PE (10^{-6} M) (Fig. 7*B*). Testosterone replacement restored the $^{45}Ca^{2+}$ efflux via the RyR both in the absence of adrenoceptor stimulation and in response to β_1-adrenoceptor stimulation with Iso (10^{-7}M) (Fig. 7, *A* and *B*).

Effects of α_1- or β_1-adrenoceptor stimulation on $^{45}Ca^{2+}$ fluxes across SERCA in ventricular myocytes isolated from control, ORX, and ORX + T rats. There were no significant differences in the rate of $^{45}Ca^{2+}$ uptake by SERCA in all groups in the absence of adrenoceptor stimulation (Fig. 8*A*). Stimulation of α_1- or β_1-adrenoceptors with PE (10^{-6} M) or Iso (10^{-7} M) increased the rate of $^{45}Ca^{2+}$ uptake by SERCA in all groups (Fig. 8*A*). The percent change in $^{45}Ca^{2+}$ uptake via SERCA was significantly greater in the control group than in the ORX group only upon administration of Iso (10^{-7} M) but not PE (10^{-6} M) (Fig. 8*B*). Testosterone replacement restored the SERCA activity (Fig. 8, *A* and *B*).

Effects of α_1- or β_1-adrenoceptor stimulation on PLB phosphorylation in ventricular myocytes isolated from control, ORX, and ORX + T rats. PLB can be phosphorylated at two adjacent sites: Ser^{16} and Thr^{17}. There were no significant differences in the

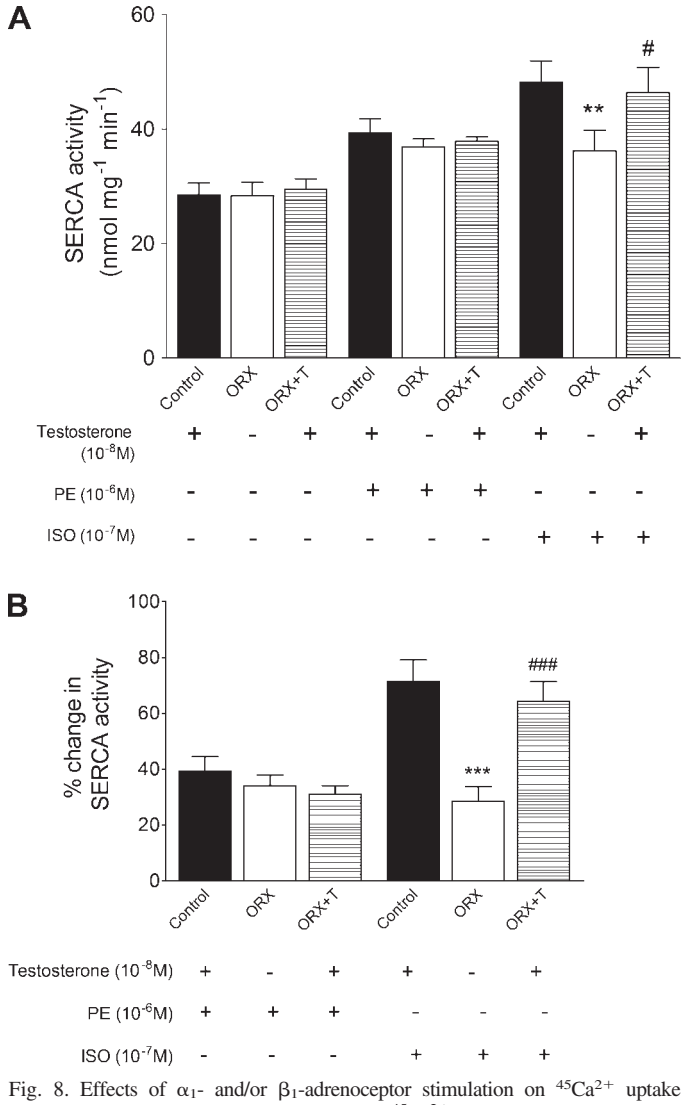


Fig. 8. Effects of α_1- and/or β_1-adrenoceptor stimulation on $^{45}Ca^{2+}$ uptake through SERCA (*A*) and the percent change in $^{45}Ca^{2+}$ uptake through SERCA (*B*) in ventricular myocytes isolated from control, ORX, and ORX + T rats. α_1- and β_1-adrenoceptors were stimulated with the selective α_1-adrenoceptor agonist PE (10^{-6} M) in the presence of Plol (10^{-6} M) or the nonselective β-adrenoceptor agonist Iso (10^{-7} M) in the presence of the β_2-adrenoceptor antagonist ICI (5×10^{-7} M) and the α-adrenoceptor antagonist Ptm (10^{-6} M). Values are means ± SE of 5–6 rats/group. $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. the corresponding control groups; $\#P < 0.05$ and $\#\#\#P < 0.001$ vs. the corresponding ORX groups.

phosphorylation of Ser^{16} residues in all groups in the presence of PE (10^{-6} M) (Fig. 9). However, phosphorylation of Thr^{17} was slightly, but significantly, lower in the ORX group than in the control and ORX + T groups in response to PE (10^{-6} M). Conversely, in the presence of Iso (10^{-7} M), phosphorylation of Ser^{16}, but not Thr^{17}, was significantly lower in the ORX group than in the control group. The effects of PE on Thr^{17} and Iso on Ser^{16} in the ORX group were restored by testosterone replacement (Fig. 9).

Effects of α_1- or β_1-adrenoceptor stimulation on $^{45}Ca^{2+}$ fluxes across sarcolemmal NCX in ventricular myocytes isolated from control, ORX, and ORX + T rats. There were no significant differences in the rate of $^{45}Ca^{2+}$ extrusion via NCX in all groups in the absence of adrenoceptor stimulation (Fig. 10*A*). Stimulation of α_1- or β_1-adrenoceptors with PE (10^{-6} M) or Iso (10^{-7} M) increased the rate of $^{45}Ca^{2+}$ extrusion via NCX in all groups (Fig. 10*A*). The percent

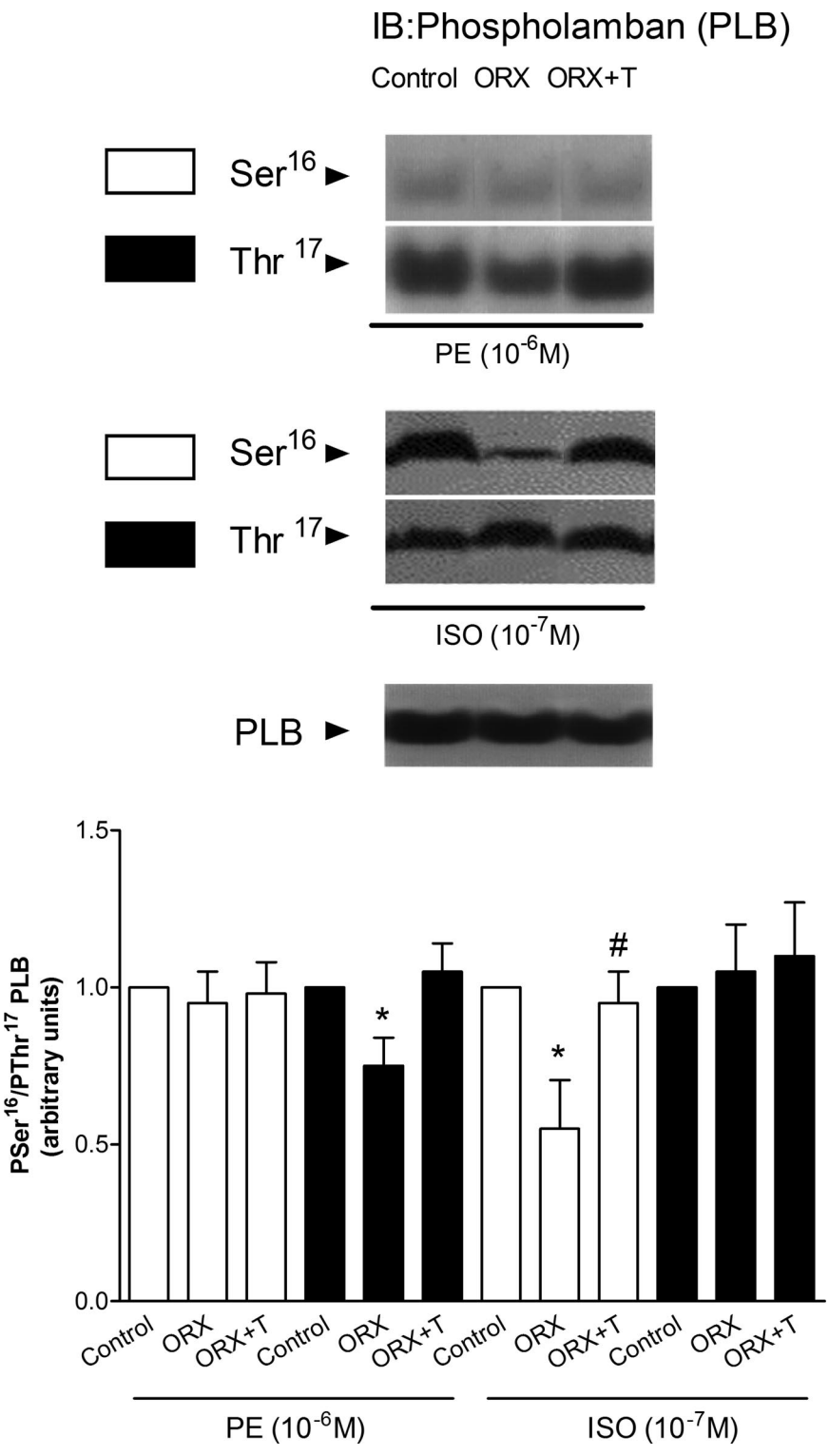


Fig. 9. Effects of α_1- or β_1-adrenoceptor stimulation on the phosphorylation of phospholamban (PLB) in ventricular myocytes isolated from control, ORX, and ORX + T rats. α_1- and β_1-adrenoceptors were stimulated with the selective α_1-adrenoceptor agonist PE (10^{-6} M) in the presence of Plol (10^{-6} M) or the nonselective β-adrenoceptor agonist Iso (10^{-7} M) in the presence of the β_2-adrenoceptor antagonist ICI (5×10^{-7} M) and the α-adrenoceptor antagonist Ptm (10^{-6} M). Values are means ± SE of 5–6 rats/group. *Top*: Western blots [immunoblots (IB)] showing phosphorylation levels of Ser^{16} and Thr^{17} in ventricular myocytes isolated from control, ORX, and ORX + T rats. *Bottom*: quantification of band intensities with respect to the control, which was given an arbitrary value of 1. Values are means ± SE of 5–6 rats/group. *$P < 0.05$ vs. the corresponding control groups; #$P < 0.05$ vs. the ORX group.

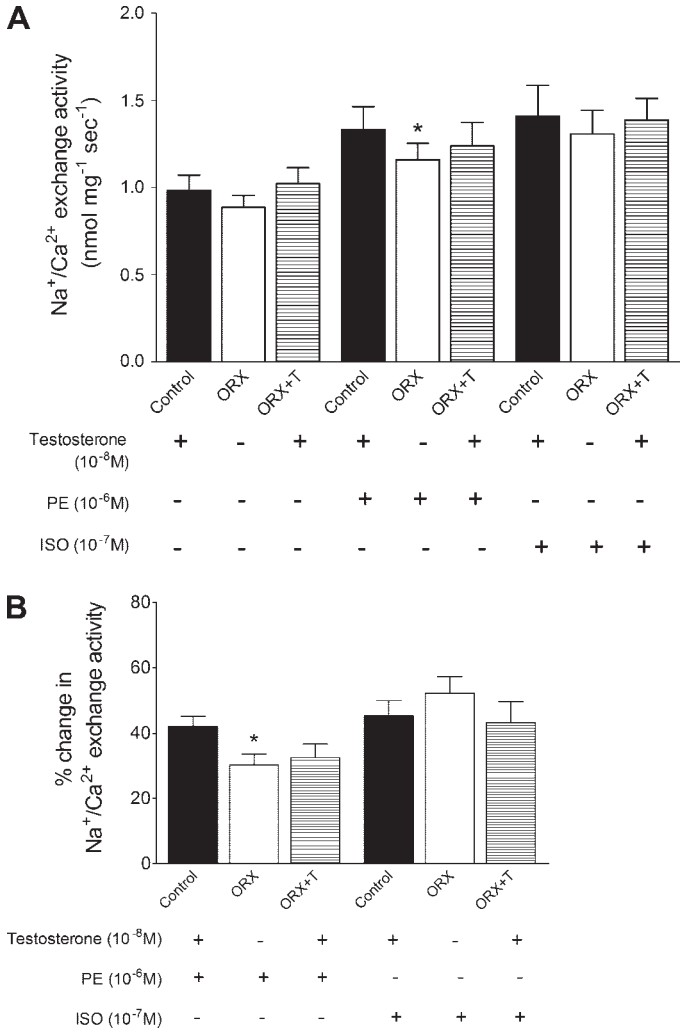


Fig. 10. Effects of α_1- and/or β_1-adrenoceptor stimulation on the extrusion of $^{45}Ca^{2+}$ by NCX (*A*) and the percent change in the extrusion of $^{45}Ca^{2+}$ by NCX (*B*) in ventricular myocytes isolated from control, ORX, and ORX + T rats. α_1- and β_1-adrenoceptors were stimulated with the selective α_1-adrenoceptor agonist PE (10^{-6} M) in the presence of Plol (10^{-6} M) or the nonselective β-adrenoceptor agonist Iso (10^{-7} M) in the presence of the β_2-adrenoceptor antagonist ICI (5×10^{-7} M) and the α-adrenoceptor antagonist Ptm (10^{-6} M). Values are means ± SE of 5–6 rats/group. *$P < 0.05$ vs. the corresponding control groups.

change in $^{45}Ca^{2+}$ extrusion via NCX was significantly greater in the control group than in the ORX group only upon administration of PE (10^{-6} M) but not Iso (10^{-7} M) (Fig. 10*B*). However, testosterone replacement did not restore the activity to normal (Fig. 10, *A* and *B*).

DISCUSSION

The present study shows that testosterone, which itself had no significant effect, potentiated the stimulation of α_1- and β_1-adrenoceptor stimulation on LVDP, $+dP/dt_{max}$, and $-dP/dt_{max}$, which indicate increased contractility and speedier relaxation, respectively. This is consistent with our recent finding showing that testosterone enhances the injury and improves contractile recovery after ischemia-reperfusion injury when these adrenoceptors are activated (55). The novel observation in the present study was that testosterone at $\sim 10^{-8}$ M markedly increased the Ca^{2+} release from the intracellular store and accelerated Ca^{2+} removal from the cytosol upon adrenoceptor stimulation. The former is related to augmented RyR activity, whereas the latter is related to enhanced NCX and SERCA activities accompanied by increased PLB phosphorylation. The results indicate that testosterone at physiological levels augmented Ca^{2+} fluxes across three Ca^{2+}-regulatory proteins, namely, the RyR, NCX, and SERCA, in response to adrenoceptor activation. Further study on the amplitude of the $C[Ca^{2+}]_i$ transient revealed that the increased amplitude of the $E[Ca^{2+}]_i$ transient may also be due at least partly to a slight, but significant, increase in releasable Ca^{2+} in the SR. Therefore, the interaction (or cross-talk) between testosterone and adrenoceptors may augment contractility and relaxation at least partly by potentiating the activity of the RyR, NCX, and SERCA and, to a lesser extent, by increasing the Ca^{2+} content in the SR. The advancement of the present study is the demonstration of the Ca^{2+} homeostasis underlying the testosterone-enhanced contractile responses to adrenoceptor stimulation.

In the present study, we found that testosterone treatment increased RyR activity, which was enhanced by adrenoceptor stimulation. This was accompanied with increases in contractility and amplitude of the $E[Ca^{2+}]_i$ transient. The observations suggest that testosterone would cause a significant increase in Ca^{2+} release from the SR during excitation-contration coupling when α_1- and/or β_1-adrenoceptors were stimulated, which accounts for the increased contractility. The increased RyR activity may also be due to increased influx of Ca^{2+} via L-type Ca^{2+} channels (17), the expression (18) and activity (15, 40) of which are enhanced by testosterone or either α_1-adrenoceptor (11–13, 34) or β_1-adrenoceptor (71) stimulation.

In a previous study, Golden et al. (6) demonstrated that testosterone increases NCX mRNA expression. However, Callies et al. (8) failed to find any effect of testosterone on the expression of SERCA 2a as well as its critical regulator PLB. In the present study, we also found no differences in the basal expression of PLB as well as SERCA and NCX activities among the three groups in the absence of adrenoceptor activation, indicating a lack of effect of testosterone on the activity of either Ca^{2+}-handling protein in the absence of adrenoceptor stimulation. On the other hand, the activities of both NCX and SERCA were significantly augmented when α_1- or β_1-adrenoceptors were activated. Furthermore, we also found that PLB phosphorylation at Ser^{16} was significantly enhanced when the β_1-adrenoceptor was activated in the presence of testosterone. This indicates that testosterone potentiated the activity of NCX to α_1-adrenoceptor stimulation and SERCA to β_1-adrenoceptor stimulation, respectively. In the latter case, the enhancing effect of testosterone on SERCA was at least in part mediated via relieving the inhibition of PLB through phosphorylation at Ser^{16}, which is known to be accessible by PKA. It is well documented that the β_1-adrenoceptor induces PKA activation and phosphorylates and relieves the inhibition of the key regulator PLB on SERCA, which promotes rapid Ca^{2+} sequestration to the SR, thus accelerating the Ca^{2+} removal. Previous studies have shown that α_1-adrenoceptor stimulation upregulates the expression of NCX (24) as well as increasing its activity (2, 41). Testosterone may therefore further amplify the Ca^{2+} removal by augmenting the effect of α_1-adrenoceptor stimulation on NCX and the effect of β_1-adrenoceptor stimulation on SERCA. In the present study, we also showed a

slightly increased phosphorylation of PLB at Thr^{17} when the α_1-adrenoceptor was activated in the presence of testosterone, which agrees with an emerging view on dual-site phosphorylation of PLB in that the phosphorylation status of Ser^{16} and Thr^{17} can be independently modulated by selective activation of PKA and Ca^{2+}-calmodulin kinase II (CAMKII), respectively, in which the phosphorylation of either site is able to hasten cardiac relaxation (22). Indeed, it has been recently demonstrated that CAMKII plays a role in the positive inotropic effect of the α_1-adrenoceptor agonist PE (21). However, we did not find enhanced activity of SERCA in response to α_1-adrenoceptor stimulation despite a slight, but significant, reduction in the phosphorylation of PLB at Thr^{17} in the present study. The slight inhibition of PLB phosphorylation at Thr^{17} by α_1-adrenoceptor stimulation may not be sufficient to elicit a significant increase in SERCA activity. Further study is warranted.

That testosterone potentiated the Ca^{2+} removal upon adrenoceptor stimulation further implicates a protective role of testosterone against myocardial injury-induced Ca^{2+} overload. Callies et al. previously demonstrated that testosterone attenuates end-ischemic Ca^{2+} overload (8), which is a precipitating cause of myocardial injury (72) and is believed to be responsible for the improved contractile recovery and reduced injury against ischemic insults (8). Myocardial ischemia triggers sympathetic overactivity, which markedly increases the release of NE (30, 50, 51) that activates both α_1- and β-adrenoceptors, leading to Ca^{2+} overload in the heart (43). Therefore, testosterone-augmented Ca^{2+} removal upon adrenoceptor stimulation may ameliorate injury after ischemia-reperfusion.

In the present study, we found that ORX did not affect LDVP, $+dP/dt_{max}$, and $-dP/dt_{max}$ in perfused hearts isolated from mature male rat in the absence of adrenoceptor stimulation. This is in agreement with observations from our previous study (35). It is also in agreement with the observation of Callies et al. (8), who did not observe any differences in the basal level of LVDP between control and ORX rats. In contrast, Scheuer et al. (49) observed decreases in stroke work, ejection fraction, fractional shortening, and velocity of circumferential fiber shortening in perfused isolated hearts of mature male rats after ORX, which was reverted by testosterone replacement. There is also evidence that ORX is accompanied by a decrease in contractility (19). The observations indicate that testosterone has a direct effect on cardiac performance. In the present study, we also found that testosterone altered Ca^{2+} handling. The T_{50} of the $E[Ca^{2+}]_i$ transient was lengthened in ventricular myocytes from ORX rats, which was abolished by testosterone replacement. The lengthening of the T_{50} may be due to decreased expression of NCX in the absence of testosterone (18). We also demonstrated a reduction of RyR activity in the ORX group despite no significant differences in the amplitude of the $E[Ca^{2+}]_i$ transient in all three groups. Indeed, there is evidence showing that testosterone increases Ca^{2+} sparks, an indication of increased release of Ca^{2+} from the SR (57). Vicencio et al. (57) recently demonstrated that testosterone induces a slow Ca^{2+} release from internal stores. Nonetheless, the basal differences in Ca^{2+} handling were not reflected in the contractile response in perfused isolated hearts. It is possible that the enhanced RyR activity as well as shortened T_{50} observed may not be great enough to result in a significant change in $+dP/dt_{max}$ and $-dP/dt_{max}$ in perfused isolated hearts. Further study is needed to address the discrepancies.

Unlike the β_1-adrenoceptor, the stimulatory role of which on cardiac contraction is well established, the effects of α_1-adrenoceptor stimulation on contractile function in the heart are controversial and depend on the species, age, and experimental conditions. A negative inotropic effect of α_1-adrenoceptor stimulation in the mouse myocardium has been reported (37). On the other hand, positive inotropic responses have been observed in rats both in vivo (6) and in vitro (14) upon α_1-adrenoceptor stimulation. In the present study, we showed that α_1-adrenoceptor stimulation with PE increased contractility and accelerated relaxation, which were accompanied, respectively, with an increased amplitude of Ca^{2+} release from the SR and faster removal of Ca^{2+} from the cytosol. These observations support a stimulatory effect of α_1-adrenoceptor activation.

In the present study, we focused on the β_1-adrenoceptor based on three lines of evidence. First, we have recently demonstrated that testosterone improves contractile recovery to stimulation of the β_1-adrenoceptor. Second, we (55) and Golden et al. (18) have demonstrated that testosterone enhances the expression of the β_1-adrenoceptor in the myocardium. Finally, in vivo and in vitro studies have shown that the β_1-adrenoceptor is the predominant subtype in the regulation of cardiac contractile functions. Nevertheless, it is well documented that activation of the β_2-adrenoceptor also increases developed contraction but does not accelerate relaxation. Using perfused isolated rat heart preparations, McConville et al. (38) reported that β_1-adrenoceptor activation caused a greater and more sustained inotropic response (LVDP, $+dP/dt_{max}$, and $-dP/dt_{max}$) than β_2-adrenoceptor activation but that only β_1-adrenoceptor, not β_2-adrenoceptor, activation leads to a positive lusitropic response. The same responses have also been shown in the rat (64, 66) and cat and sheep (5, 32). In agreement with this finding, Xiao et al. (64, 67) showed that the β_2-adrenoceptor differs from the β_1-adrenoceptor by activating a specific signaling pathway to change $[Ca^{2+}]_i$ and twitch. Zinterol, a β_2-adrenoceptor-selective agonist, increases the amplitude of both $[Ca^{2+}]_i$ and twitch as well as inward Ca^{2+} current without changing the time course of $[Ca^{2+}]_i$ and twitch duration in rats (64). Our preliminary results showed that testosterone also upregulated the β_2-adrenoceptor in buoyant membrane fractions (unpublished observations). The role of the β_2-adrenoeptor in testosterone-potentiated contractile responses and the underlying Ca^{2+} homeostasis needs further investigation.

We found that the effects of testosterone were abolished by blockade of the receptor with Cyp, a selective androgen receptor antagonist, indicating an androgen receptor-mediated effect, which is mostly genomic. There is, however, increasing evidence showing that testosterone also acutely increases contractility via an androgen receptor-independent nongenomic pathway. Vicencio et al. (57) demonstrated that acute exposure to testosterone rapidly increases $[Ca^{2+}]_i$, the rise of which is not inhibited by Cyp. This may at least partly explain why only a partial blockade of the $E[Ca^{2+}]_i$ transient was achieved by Cyp.

It is well established that the β_1-adrenoceptor plays the predominant role in the regulation of cardiac contraction. In support of this, we also showed that the effects of testosterone to α_1-adrenoceptor stimulation were smaller than the corresponding values with the β_1-adrenoceptor (supplemental material).[1]

[1] Supplemental material for this article is available online at the *American Journal of Physiology-Cell Physiology* website.

Nevertheless, the functional responsiveness of the cardiac β-adrenoceptor is diminished with aging, which is associated with a decrease in β_1-adrenoceptor density in the ventricular myocardium (61) and a decrease in the catalytic unit of adenylyl cyclase in the atria (7), which diminish cAMP formation upon β-adrenoceptor stimulation. The fact that testosterone potentiated α_1-adrenoceptor-mediated positive inotropy and lusitropy indicates that testosterone may therefore play a more important role in such situations to regulate contractile function. The enhancing effects of testosterone should be taken into consideration in the treatment of contractile dysfunction, particularly in the male aging population, which has lower testosterone (25, 27).

In conclusion, we observed that testosterone potentiated the stimulatory effects of both α_1- and β_1-adrenoceptors on contractile function of the heart, namely, increased contractility and speedier relaxation, which were due to increased release of Ca^{2+} from the SR and a more efficient removal of Ca^{2+} from the cytosol, respectively. The former was, in turn, due to increased Ca^{2+} release from the SR via the RyR, whereas the latter was due to more efficient Ca^{2+} reuptake to the SR through SERCA via increased phosphorylation of PLB in the case of β_1-adrenoceptor stimulation and more efficient Ca^{2+} extrusion via sarcolemmal NCX in the case of α_1-adrenoceptors stimulation.

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