Testosterone Induces an Intracellular Calcium Increase by a Nongenomic Mechanism in Cultured Rat Cardiac Myocytes

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Androgens are associated with important effects on the heart, such as hypertrophy or apoptosis. These responses involve the intracellular androgen receptor. However, the mechanisms of how androgens activate several membrane signaling pathways are not fully elucidated. We have investigated the effect of testosterone on intracellular calcium in cultured rat cardiac myocytes. Using fluo3-AM and epifluorescence microscopy, we found that exposure to testosterone rapidly (1–7 min) led to an increase of intracellular Ca^{2+}, an effect that persisted in the absence of external Ca^{2+}. Immunocytochemical analysis showed that these effects occurred before translocation of the intracellular androgen receptor to the perinuclear zone. Pretreatment of the cells with 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid-acetoxymethylester and thapsigargin blocked this response, suggesting the involvement of internal Ca^{2+} stores. U-73122, an inhibitor of phospholipase C, and xestospongin C, an inhibitor of inositol 1,4,5-trisphosphate receptor, abolished the Ca^{2+} signal. The rise in intracellular Ca^{2+} was not inhibited by cyproterone, an antagonist of intracellular androgen receptor. Moreover, the cell impermeant testosterone-BSA complex also produced the Ca^{2+} signal, indicating its origin in the plasma membrane. This effect was observed in cultured neonatal and adult rat cardiac myocytes. *Pertussis* toxin and the adenoviral transduction of β-adrenergic receptor kinase carboxy terminal peptide, a peptide inhibitor of βγ-subunits of G protein, abolished the testosterone-induced Ca^{2+} release. In summary, this is the first study of rapid, nongenomic intracellular Ca^{2+} signaling of testosterone in cardiac myocytes. Using various inhibitors and testosterone-BSA complex, the mechanism for the rapid, testosterone-induced increase in intracellular Ca^{2+} is through activation of a plasma membrane receptor associated with a *Pertussis* toxin-sensitive G protein-phospholipase C/inositol 1,4,5-trisphosphate signaling pathway.

CARDIAC MUSCLE is a target for the action of endogenous anabolic androgenic steroids (AAS) (1) and synthetic derivatives of the hormone testosterone (2). AAS have been used by athletes to improve performance by increasing muscle mass and strength. Among the numerous documented toxic and hormonal effects of AAS, attention has been recently focused especially on the cardiovascular effects (3). It is known that there are increases in blood pressure and peripheral arterial resistance (4, 5), and there are also effects on the heart muscle, primarily left ventricular hypertrophy with restricted diastolic function (6–8). Severe cardiac complications (heart failure, ventricular fibrillation, ventricular thromboses, myocardial infarction, or sudden cardiac death) in individual strength athletes with acute AAS abuse have also been reported (3, 9, 10). These processes involve changes in gene expression controlled by intracellular androgen receptor-mediated pathways. Recent studies have, however, demonstrated alternative, rapid intracellular androgen receptor-independent mode of testosterone action. For example, administration of testosterone acutely induces vasodilatation in the systemic, coronary, and pulmonary vascular beds (11). Acting in this way, testosterone might increase myocardial tolerance to ischemia. Testosterone replacement in orchidectomized rats improved the recovery of myocardial function after ischemia/reperfusion injury (12). Er *et al.* (13) have recently shown that testosterone is directly cytoprotective in the myocardium through activation of ATP-sensitive K^+ channels in the mitochondrial inner membrane.

Similar nongenomic effects are known in several cell types, including rat osteoblasts (14), macrophages (15), and skeletal muscle cells (16). It has been proposed that these rapid androgen actions may be exerted through membrane receptors that stimulate early intracellular signaling pathways through interaction with G proteins (17, 18). Common to these early effects are the fast intracellular Ca^{2+} increase, activation of Ca^{2+}-dependent pathways and second-messenger cascades (19). In T cells, membrane androgen receptor mediates ligand-induced Ca^{2+} influx through nonvoltage-gated, Ni^{2+}-blockable Ca^{2+} channels (18, 20). In osteoblasts, testosterone stimulates both the influx of extracellular Ca^{2+} via voltage-

Abbreviations: AAS, Anabolic androgenic steroids; Ad-βARKct, adenovirus overexpressing βARKct; Ad-EV, empty adenoviral construct; βARKct, β-adrenergic receptor kinase carboxy terminal peptide; BAPTA-AM, 1,2-bis(2-amino-phenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid-acetoxymethylester; Ca^{2+} calcium; $\Delta F/F_0$, relative total fluorescence; FITC, fluorescein isothiocyanate; fluo3-AM, fluo3 acetoxymethylester; IP_3, inositol 1,4,5-trisphosphate; IP_3R, IP_3 receptor; MOI, multiplicity of infection; PLC, phospholipase C; PTX, *Bordetella pertussis* toxin; RF max, maximum values of relative fluorescence; T-BSA, testosterone covalently bound to albumin.

gated Ca^{2+} channels and Ca^{2+} release from intracellular stores through G protein-coupled receptors activating phospholipase C (PLC) via a *Bordetella pertussis* toxin (PTX)-sensitive G protein (14). Murine macrophages respond to testosterone by predominantly intracellular Ca^{2+} mobilization mediated through G protein-coupled receptors for testosterone (15, 21). We have previously shown that, in skeletal muscle cells, both testosterone and nandrolone produce rapid intracellular Ca^{2+} transients (16) involving Ca^{2+} release from inositol 1,4,5-trisphosphate (IP_3)-sensitive stores (22). This rapid Ca^{2+} transient was not modified by cyproterone, an inhibitor of steroid binding to its intracellular androgen receptor. Ca^{2+} signals elicited by steroid hormones through an increase in intracellular IP_3 and associated with increased nucleoplasmic Ca^{2+} may have an important role in the regulation of different processes in the muscle cell (23).

In cardiac myocytes, intracellular Ca^{2+} regulates contraction, but an alternative role for Ca^{2+} in these cells as a regulator of gene expression has been proposed (17, 19). Cardiac myocytes contain intracellular androgen receptors (24), which regulate the expression of several genes (25, 26). In this work, we report the early effects of testosterone on intracellular Ca^{2+} in cultured cardiac myocytes. Our results show that, in cultured cardiac myocytes, testosterone induces a rapid and nongenomic intracellular Ca^{2+} release through activation of a plasma membrane androgen receptor associated with the PTX-sensitive G protein-PLC/IP_3 signaling pathway.

Materials and Methods

Materials

Testosterone, testosterone-BSA, BSA, cyproterone acetate (6-chloro-1β,2β-dihydro-17-hydroxy-3′H-cyclopropa[1, 2]pregna-1,4,6-triene-3,20-dione acetate), medium 199, DMEM, PTX, IP_3, ryanodine, thapsigargin, and U-73122 were purchased from Sigma-Aldrich Corp. (St. Louis, MO). [^{3}H]IP_3 was from DuPont NEN Life Science Products (Boston, MA). Fluo3-acetoxymethylester (fluo3-AM) and 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid-acetoxymethylester (BAPTA-AM) were purchased from Molecular Probes (Eugene, OR). Genistein and xestospongin C were from Calbiochem (La Jolla, CA). Fluorescein isothiocyanate (FITC)-conjugated goat antirabbit IgG was from Pierce Chemical Co. (Rockford, IL). Intracellular androgen receptor antibody (C-19) was from Santa Cruz Biotechnology (Santa Cruz, CA). All other reagents or molecular biology grade chemicals were obtained from Sigma Chemical Co.

Culture of cardiac myocytes

Neonatal rat cardiac myocytes were prepared from hearts of 1- to 3-d-old Sprague Dawley rats as described previously (27). For determination of IP_3 and intracellular Ca^{2+}, cardiac myocytes were plated at a final density of $0.7 \times 10^3/mm^2$ on gelatin-precoated-60 mm dishes and $1.0 \times 10^3/mm^2$ on gelatin-precoated coverslips, respectively. Serum was withdrawn 24 h before cells were used. To prevent the overgrowth of fibroblast and smooth muscle cells, 10 μM bromodeoxyuridine was used in our cell culture media. Cultured cardiac myocytes were identified using an anti-β-myosin heavy-chain antibody. Cell cultures were at least 95% pure. Freshly dispersed adult rat cardiac myocytes were prepared from hearts of male adult Sprague Dawley rats (>250 g). Rats were anesthetized with ketamin and xylazin, hearts were removed, washed with Gerard buffer [0.19 mM NaH_2PO_4, 1.01 mM Na_2HPO_4, 10 mM HEPES, 128 mM NaCl, 4 mM KCl, 1.4 mM $MgSO_4$, 5.5 mM glucose, 2 mM pyruvic acid (pH 7.4)] and retroperfused at 4 ml/min with 1 mM $CaCl_2$ containing Gerard buffer during 5 min, followed by 1 mM EGTA containing Gerard buffer during 1 min and finally with 0.07% (wt/vol) collagenase and hyaluronidase containing Gerard buffer (digestion solution) for 30 min. Digested hearts were mechanically shattered in 5 ml Gerard buffer, mixed with 20 ml digestion solution, incubated at 37 C with constant agitation for 10 min, and supernatants centrifuged at 500 rpm for 30 sec. Remaining tissue was further digested with 20 ml digestion solutions. Pellets containing cardiac myocytes were resuspended in Gerard buffer and plated to obtain a final density of $1.0 \times 10^3/mm^2$ on laminin-precoated coverslips. Rats were from the Animal Breeding Facility from the Faculty of Chemical and Pharmaceutical Sciences, University of Chile (Santiago, Chile). The Animal Investigation Committee approved all experimental procedures involving animals. This investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (publication no. 85–23, revised 1985).

Adenovirus and inhibitors

Adenoviral vectors were propagated and purified as previously described (28). Two transgenes (a gift from Dr. W. J. Koch, Duke University Medical Center, Durham, NC) were used: adenovirus (Ad-) overexpressing β-adrenergic receptor kinase carboxy terminal peptide (βARKct) and an empty adenoviral construct (Ad-EV). βARKct is a peptide inhibitor of G$\beta\gamma$ signaling (29). Cardiac myocytes were transduced with adenoviral vectors at a multiplicity of infection (MOI) of 300 and incubated 24 h a 37 C in DMEM/medium 199. Cardiac myocytes were preincubated for 30 min before addition of the androgen with BAPTA-AM (100 μM), U-73122 (50 μM), U-73343 (50 μM), xestospongin C (100 μM), ryanodine (20 μM), cyproterone (1 μM), or genistein (50 μM) or for 6 h with PTX (1 μg/ml).

Intracellular calcium

Intracellular Ca^{2+} measurements were obtained from cardiac myocytes preloaded with the fluorescent Ca^{2+}-sensitive dye fluo3-AM, using a fluorescence microscope (Olympus Diaphot-TMD, Nikon Corp., Tokyo, Japan) equipped with a cooled charge-coupled device camera and an image acquisition system (MCD 600 Spectra Source, Westlake Village, CA). Cardiac myocytes were washed three times with Ca^{2+}-containing media (145 mM NaCl, 5 mM KCl, 2.6 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES-Na, 5.6 mM glucose, adjusted to pH 7.4 with HCl) and loaded with 5.4 μM fluo3-AM (diluted from a 1.08 mM stock in 20% pluronic acid-dimethylsulfoxide) for 30 min at room temperature. After dye loading, cardiac myocytes were washed either with the Ca^{2+}-containing medium or with a Ca^{2+}-free medium (145 mM NaCl, 5 mM KCl, 1.0 mM EGTA, 3.6 mM $MgCl_2$, 10 mM HEPES-Na, 5.6 mM glucose, adjusted to pH 7.4 with HCl) and used for experiments. Coverslips with adherent cells were mounted in a 1-ml-capacity plastic chamber and placed in the microscope for fluorescence measurements. Testosterone was either added directly or through rapid changing of medium (1 sec). The fluorescent images were collected every 2.0–5.0 sec and analyzed frame by frame with an image data acquisition program (Spectra Source). An objective lens PlanApo ×60 (numerical aperture 1.4) was generally used. Most images were acquired at 512 × 120 pixels. Intracellular Ca^{2+} levels were expressed as relative total fluorescence [$\Delta F/F_0$: ratio of fluorescence difference, stimulated-basal (F_i-F_0), to basal value (F_0)] as a function of time. The fluorescence intensity increases proportionally with intracellular Ca^{2+} (30).

Digital image processing

Elimination of out-of-focus fluorescence was performed using both the no-neighbors deconvolution algorithm and Castleman's (31) point spread function theoretical model, as described previously (16). For quantification of fluorescence, the summed pixel intensity was calculated from the section delimited by a contour. As a way of increasing efficiency of these data manipulations, action sequences were generated. To avoid interference from possible testosterone effects on the cellular volume, the area of each fluorescent cell was determined by image analysis using an adaptive contour and then creation of a binary mask, which was compared with its bright-field image.

Measurement of IP_3 levels

Cardiac myocytes were rinsed and preincubated for 20 min at room temperature in 58 mM NaCl, 4.7 mM KCl, 3 mM $CaCl_2$, 1.2 mM $MgSO_4$,

0.5 mM EDTA, 60 mM LiCl, 10 mM glucose, and 20 mM HEPES, adjusted to pH 7.4 with HCl. Cells were stimulated by rapid addition of testosterone (final concentration 100 nM). At the indicated times, reactions were stopped by aspiration of the stimulating solution, addition of 0.8 M ice-cold perchloric acid and freezing with liquid nitrogen. Samples were allowed to thaw and cell debris was spun down for protein determination. Supernatants were neutralized with a solution of 2 M KOH, 0.1 M 2-(*N*-morpholine) ethane sulfonic acid, and 15 mM EDTA. The neutralized extracts were frozen at −80 C until required for IP_3 determination. Measurements of IP_3 mass were made by a validated radioreceptor assay (32). Briefly, a crude rat cerebellar membrane preparation was obtained after homogenization of tissue in 50 mM Tris-HCl (pH 7.7) containing 1 mM EDTA, 2 mM β-mercaptoethanol, and centrifugation at 20,000 × *g* for 15 min. This procedure was repeated three times, suspending the final pellet in the same solution plus 0.3 M sucrose and freezing it at −80 C until required for use. The rat cerebellar membrane preparation was calibrated for IP_3 binding with 1.6 nM [^{3}H]IP_3 and 2–120 nM cold IP_3, with sample analysis performed in a similar way but replacing cold IP_3 with a portion of the neutralized supernatant. [^{3}H]IP_3 radioactivity, which remained bound to membranes, was measured in an LS-6000TA liquid scintillation spectrometer (Beckman Instruments Corp., Fullerton, CA). Protein was determined by the Lowry method (33).

Immunocytochemistry

Intracellular androgen receptor was localized using indirect immunofluorescence. Testosterone-stimulated and nonstimulated cardiac myocytes were washed three times with PBS and then fixed with 100% methanol at −20 C for 20 min and treated with a blocking solution of 1% BSA in PBS for 30 min. Cells were incubated with the primary polyclonal antiintracellular androgen receptor antibody (1:100) overnight at 4 C. Later cardiac myocytes were washed in PBS and incubated with FITC-conjugated goat antirabbit IgG diluted 1:200 for 2 h at room temperature. Cells were washed and Vectashield (Vector Laboratories Inc., Burlingame, CA) was added to prevent bleaching. Cardiac myocytes were examined with a confocal microscope (135-M LSM Microsystems, Carl Zeiss AG, Oberkochen, Germany). Controls were performed as previously described (22).

Expression of results and statistical analysis

Data are means ± SE of the number of independent experiment indicated in the figure legends or are representative experiments performed on at least three separate occasions with similar outcomes. Data were analyzed by ANOVA, and comparisons between groups were performed using a protected Dunnett's test. A value of $P < 0.05$ was set as the limit of statistical significance.

Results

Effect of testosterone on intracellular Ca^{2+} levels in cultured cardiac myocytes

Cardiac myocytes were preloaded with fluo3-AM, and the relative fluorescence $\Delta F/F_0$ of single cells for each series of images was calculated. Cardiac myocytes maintained in Ca^{2+}-containing medium showed basal oscillations of intracellular Ca^{2+} (Fig. 1A). Accordingly, Ca^{2+} oscillations were associated with Ca^{2+} mobilization due to spontaneous contraction of the myocytes. Testosterone stimulation (100 nM) did not modify the oscillation frequency of cardiac myocytes maintained in Ca^{2+}-containing medium. The amplitude of oscillation varied from cell to cell, but the frequency was relatively constant at 0.12 ± 0.05 Hz (see Fig. 1, C and D). A sequence of fluorescence images showing intracellular Ca^{2+} oscillations in cardiac myocytes stimulated with testosterone (100 nM) is shown in Fig. 1B. Ryanodine (20 μM) completely abolished both spontaneous and testosterone-induced intracellular Ca^{2+} oscillations (Fig. 1, A and C), suggesting that

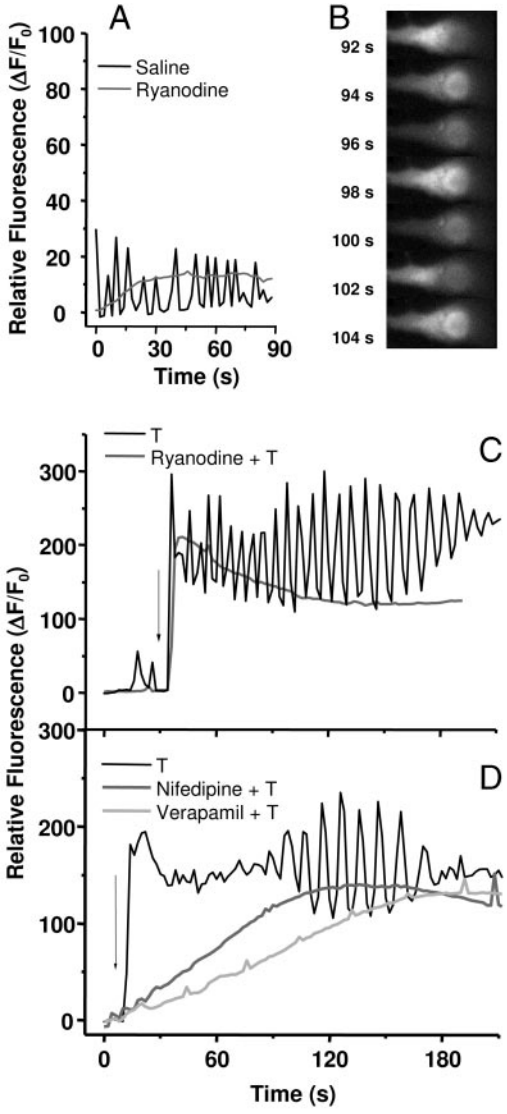

FIG. 1. Testosterone increases intracellular Ca^{2+} levels in cultured rat cardiac myocytes maintained in Ca^{2+}-containing medium. Cells were preloaded with fluo3-AM and maintained in Ca^{2+}-containing medium at the moment of stimulation. A, Intracellular Ca^{2+} level oscillations in nonstimulated cells (*black line*) and pretreated with ryanodine (20 μM, *gray line*). B, Series of images showing intracellular Ca^{2+} level oscillations in cultured cardiac myocytes treated with testosterone (100 nM). C, $\Delta F/F_0$ was calculated from fluorescence images in B (*black line*) and those obtained from cardiac myocytes preincubated with ryanodine (20 μM, *gray line*) and stimulated with testosterone. D, $\Delta F/F_0$ calculated from fluorescence images of cardiac myocytes treated with testosterone (100 nM, *black line*) or preincubated with nifedipine (10 μM, *gray line*) or verapamil (10 μM, *light gray line*), and then stimulated with testosterone, respectively. Each graph is representative from triplicates of at least three independent experiments. *Arrows* indicate the time of addition of testosterone.

ryanodine receptors were involved. This last result is consistent with the observation that Ca^{2+} oscillations are associated with contraction. It has been described that androgens inhibited Ca^{2+} influx by L-type Ca^{2+} channels in smooth muscle cells (34–36). To evaluate this possibility, cardiac myocytes were preincubated with L-type Ca^{2+} channel blockers (nifedipine or verapamil) and then stimulated with

testosterone (100 nM) in Ca^{2+} containing medium. As shown in Fig. 1D, both L-type channel blockers have similar effects, eliminating fast influx of Ca^{2+} and Ca^{2+} oscillations in cultured cardiac myocytes treated with testosterone.

When experiments were performed in Ca^{2+}-free medium, no Ca^{2+} oscillations were detected in both basal and testosterone-treated cardiac myocytes. Figure 2A shows a representative sequence of images depicting the effect of testosterone on intracellular Ca^{2+} levels in cardiac myocytes maintained in Ca^{2+}-free medium. $\Delta F/F_0$ calculated from Fig. 2A showed that intracellular Ca^{2+} levels increased slower than in Ca^{2+}-containing medium, reaching a maximum at approximately 3 min (Fig. 2B). The kinetics of the increases in nuclear and cytosolic Ca^{2+} was similar (Fig. 2C). A dose-response relationship was observed from 0 to 1 μM testosterone on the maximum values of relative fluorescence (RF max) (Fig. 2D). Maximal effect was obtained at 100 nM (Fig. 2D), and this concentration was chosen for all experiments.

Effect of testosterone on intracellular Ca^{2+} stores in cultured cardiac myocytes

The persistence of the Ca^{2+} signal in experiments with Ca^{2+}-free medium was consistent with testosterone stimulating Ca^{2+} mobilization from intracellular stores. To determine the participation of internal Ca^{2+} stores in the testosterone-induced intracellular Ca^{2+} increase, cardiac myocytes were pretreated with BAPTA-AM before testosterone treatment. This compound eliminated the testosterone-induced signal observed in absence of external calcium (Fig. 3A). After depletion of intracellular Ca^{2+} stores with the specific sarcoendoplasmic reticulum Ca^{2+}-ATPase pump inhibitor thapsigargin, testosterone no longer elicited the Ca^{2+} signal in cells incubated in Ca^{2+}-free medium (Fig. 3B). Ryanodine did not abolish the Ca^{2+} signal induced by testosterone in cells maintained in Ca^{2+}-free medium (Fig. 3C), indicating that ryanodine receptors do not participate in the slower component of the testosterone-induced calcium signal, which involves Ca^{2+} release from intracellular stores.

To investigate the role of the PLC-IP_3-IP_3 receptor (IP_3R) signaling pathway on the increases in intracellular Ca^{2+} levels induced by testosterone, cardiac myocytes were pretreated with U-73122 (a general PLC inhibitor) or xestospongin C (an IP_3R blocker). As shown in Fig. 4, A and C, both compounds abolished the testosterone effect on intracellular Ca^{2+} levels. There are some reports indicating that U-73122 may alter intracellular Ca^{2+} responses by PLC-independent effects (37–40). U-73343 an isomer that is inactive as a PLC inhibitor was used as control. Figure 4B depicts that U-73343 did not modify the effect of testosterone on the intracellular Ca^{2+} levels. To further demonstrate that testosterone increases intracellular IP_3 levels, cultured cardiac myocytes were exposed to this hormone at different times in Ca^{2+}-free medium and IP_3 contents were quantified by a radioreceptor assay. Figure 4D shows that testosterone significantly increased IP_3 levels after 15 sec, reaching a maximum at 30 sec and then decreasing after 3 min. Collectively, these experiments suggest that the PLC-IP_3-IP_3R signaling pathway is involved in the testosterone induced Ca^{2+} release from intracellular stores.

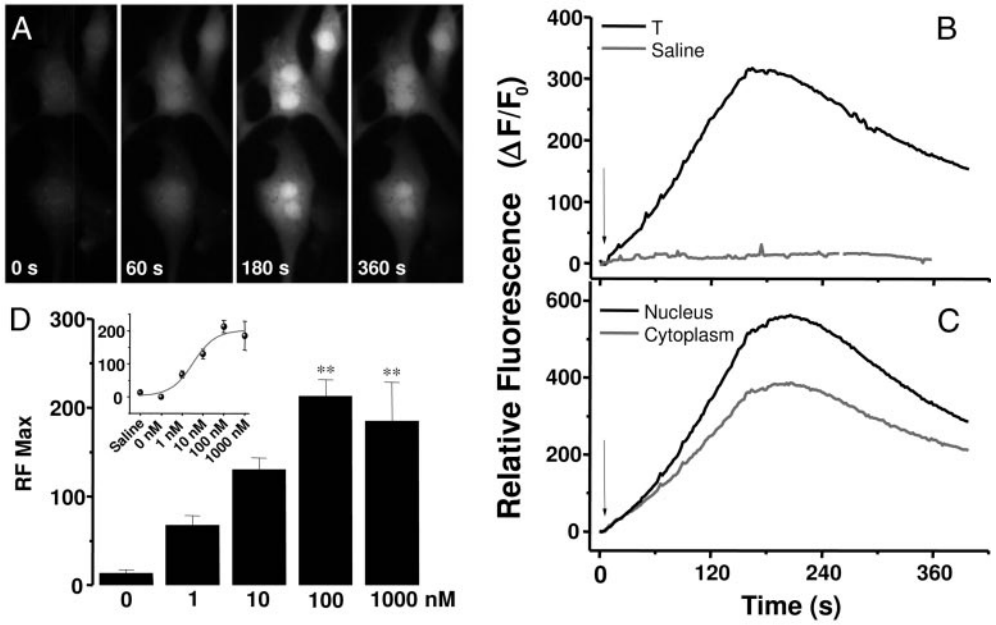

FIG. 2. Testosterone (T) effect on intracellular Ca^{2+} levels in cultured cardiac myocytes maintained in Ca^{2+}-free medium. Cells were preloaded with fluo3-AM and maintained in Ca^{2+}-free medium at the moment of stimulation. A, Serial of fluorescence images at the indicated times showing intracellular Ca^{2+} level changes in cardiac myocytes stimulated with 100 nM testosterone. B, $\Delta F/F_0$ calculated from the fluorescence images shown in A (*black line*), corresponding to cells treated with testosterone and from cells treated with saline solution (*gray line*). C, Nuclear (*black line*) and cytosolic (*gray line*) region of interest (ROI) analysis of the testosterone-induced Ca^{2+} signal from A. D, Concentration-dependent response. The graphic shows a statistical analysis of the RF max from all experiments performed with cardiac myocytes treated with testosterone or saline solution. The *inset graphic* is presented in logarithmic scale. Values are expressed as mean ± SE of triplicates from three independent experiments. **, $P < 0.01$ *vs.* 0 nM (saline). *Arrows* indicate the time of addition of testosterone.

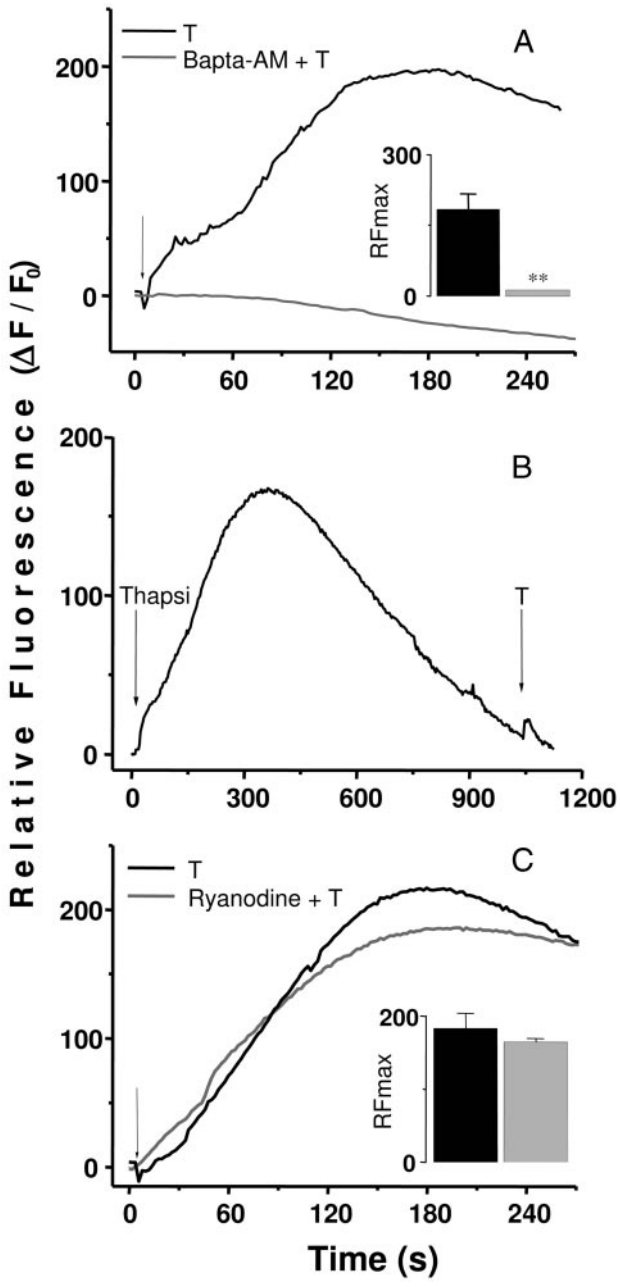

FIG. 3. Effect of BAPTA-AM, thapsigargin, and ryanodine on the testosterone (T) effect on intracellular Ca^{2+} levels. Cardiac myocytes were preloaded with fluo3-AM and maintained in Ca^{2+}-free medium at the moment of stimulation. A, Total $\Delta F/F_0$ calculated from fluorescence images of cardiac myocytes treated with testosterone (100 nM, *black line*) or preincubated with BAPTA-AM (100 μM for 30 min, *gray line*) and then stimulated with testosterone. B, Total $\Delta F/F_0$ calculated from fluorescence images of cardiac myocytes treated with thapsigargin (1 μM) for approximately 18 min to deplete intracellular calcium stores and then stimulated with testosterone (100 nM), as indicated by the respective *arrows*. C, Total $\Delta F/F_0$ calculated from images obtained from cells treated with testosterone 100 nM (*black line*) or preincubated with ryanodine (20 μM, *gray line*) and stimulated with testosterone. The *inset graphics* represent the statistical analysis of maximum relative fluorescence from the experiments performed with the respective inhibitors. Values are expressed as mean ± SE of duplicates from at least three independent experiments. **, $P < 0.01$ *vs.* control (testosterone). *Arrows* (A and C) indicate the time of addition of testosterone.

Participation of a membrane receptor in the testosterone-induced Ca^{2+} transients in cultured cardiac myocytes

Androgens exert their genomic effects by binding to intracellular receptors and then translocate as a hormone-receptor complex to the nucleus (41). The intracellular androgen receptor antagonist cyproterone inhibits genomic activation in several cell types (42). Immunocytochemical experiments for intracellular androgen receptor were performed in cultured rat cardiac myocytes at different times of stimulation with testosterone. In control cells (Fig. 5A), intracellular androgen receptor was mainly found in the cytosol, a condition that was not modified after 5 min of testosterone treatment (Fig. 5B). Only after 60 min was there a decrease in the cytosolic distribution of the intracellular androgen receptor (Fig. 5C). The subcellular distribution of the androgen receptor was mainly perinuclear after 2 h of stimulation with testosterone (Fig. 5D). Cyproterone (1 μM for 30 min) had no effect on the distribution of the androgen receptor in nonstimulated cardiac cells (Fig. 5E) but completely blocked testosterone-induced translocation of the intracellular receptor (Fig. 5F), despite being unable to prevent the effect of on the Ca^{2+} signal (Fig. 6A). The time course for intracellular androgen receptor translocation induced by testosterone was also clearly longer than the time necessary for the testosterone-dependent increase in intracellular Ca^{2+} levels. It is unlikely, therefore, that the intracellular androgen receptor plays any role in the short-term testosterone effects.

To further investigate the early signaling events triggered by testosterone on cardiac myocytes related to the effect on intracellular Ca^{2+} levels, cells were pretreated with genistein (50 μM for 30 min) or PTX (1 μg/ml for 2 h) and then stimulated with testosterone in absence of external Ca^{2+}. Genistein did not modify the testosterone-induced Ca^{2+} signal, whereas with PTX, it was completely abolished (Fig. 6B). Ad-βARKct, a peptide binding to and inactivating the $\beta\gamma$-subunits of G protein (28, 29), inhibited the testosterone-induced Ca^{2+} transients; control vector had no effect (Fig. 6C). These results indicated that a PTX-sensitive heterotrimeric G protein is a critical component of the testosterone-induced Ca^{2+} release in cardiac myocytes.

The participation of membrane steroid receptor on the different nongenomic steroid effects, including those on intracellular Ca^{2+} levels, has been proposed in many cell types (43–45). To further evaluate this possibility, testosterone covalently bound to albumin (T-BSA), which cannot enter the cell and exerts effects only at the cell membrane, was used. T-BSA did not change the distribution of intracellular androgen receptor after 2 h of stimulation as did the free hormone (Fig. 5G) but reproduced the Ca^{2+} increase seen with free testosterone. Moreover, control experiments with BSA alone were without effect (Fig. 7A). As shown in Fig. 7B, U-73122, xestospongin C, PTX, and Ad-βARKct, but not cyproterone, blocked the effect of T-BSA on intracellular Ca^{2+} levels. These results suggest that testosterone affects intracellular Ca^{2+} levels through binding to a plasma membrane androgen receptor. To exclude any unspecific effect of testosterone on the cell membrane, which may lead to changes on intracellular Ca^{2+} levels, Δ^4-androstene-3,7-dione (a metabolic precursor with reduced androgen activity)

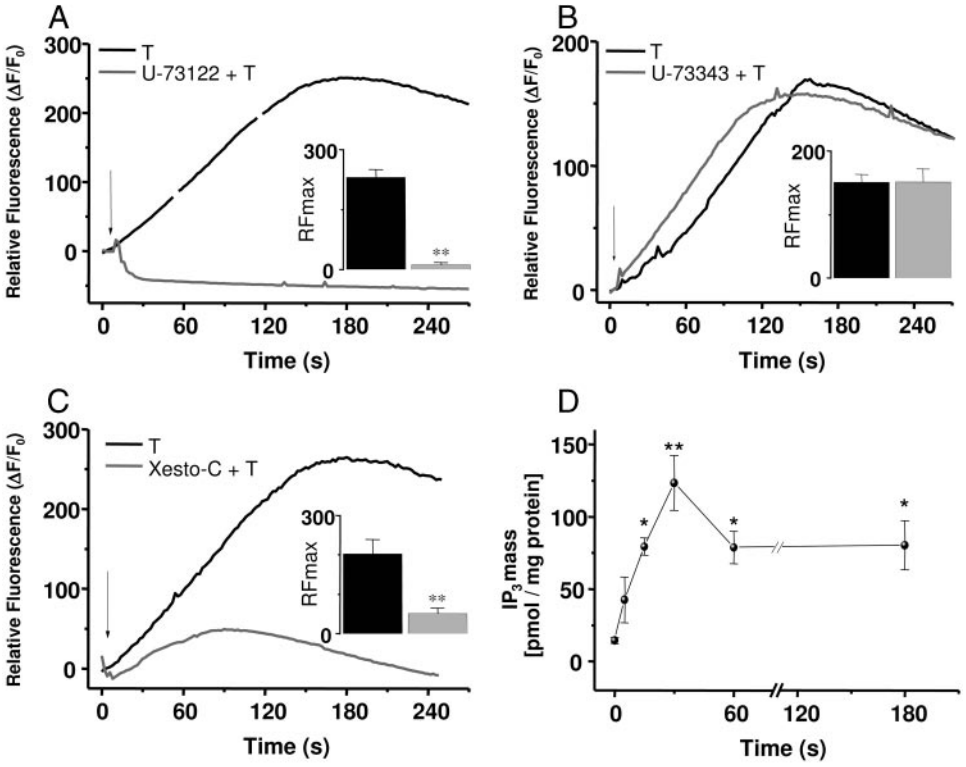

FIG. 4. Testosterone (T) activates the PLC/IP_3 pathway in cultured cardiac myocytes. Cells were preloaded with fluo3-AM, treated for 30 min with U-73122 (50 μM, A), U-73343 (50 μM, B), or xestospongin C (100 μM, C) and stimulated with testosterone 100 nM in Ca^{2+}-free resting medium. Total $\Delta F/F_0$ was calculated from fluorescence images. The *inset graphics* (A–C) correspond to the statistical analysis of RF max from experiments with the respective inhibitors and testosterone. Values are expressed as mean ± SE of triplicates from at least three independent experiments. **, $P < 0.01$ *vs.* controls. D, Cells were stimulated with 100 nM testosterone, and at the indicated times, IP_3 mass was determined by a radioreceptor assay. Values are the mean ± SE of three independent experiments. **, $P < 0.01$; *, $P < 0.05$ *vs.* time 0.

was used. Figure 7C shows that Δ^4-androstene-3,7-dione also increased intracellular Ca^{2+} levels but with a weaker and not significant response in comparison with testosterone. To further evaluate the effect of testosterone in completely differentiated cardiac myocytes, we performed the same epifluorescence studies using adult rat cardiac myocytes. In the absence of extracellular calcium, testosterone (100 nM) elicited a similar response in intracellular calcium (Fig. 8); moreover, T-BSA at the same concentration mimicked the effect of the free hormone.

Discussion

This is the first study describing an intracellular Ca^{2+} increase in response to testosterone in cardiac myocytes. This short-term, nongenomic effect of testosterone has been previously observed in other cell types (14, 20, 22, 44). Moreover, the increase in intracellular Ca^{2+} levels was dependent on Ca^{2+} release from intracellular stores by a PLC/IP_3-dependent mechanism. This effect was not linked to the intracellular androgen receptor but to a putative plasma membrane androgen receptor associated with a PTX-sensitive G protein.

The different time course of testosterone-induced intracellular Ca^{2+} signals, observed in cardiac myocytes maintained with and without external Ca^{2+}, suggests that at least two Ca^{2+} components were involved. Results showed that testosterone induces a fast Ca^{2+} influx, followed by a slower Ca^{2+} release from intracellular stores. The maximal effect on intracellular Ca^{2+} levels was obtained with 100 nM testosterone, a circulating concentration that mimics a pathophysiological state detected on individuals using the hormone therapeutically (46, 47).

Basal intracellular Ca^{2+} oscillations associated to external Ca^{2+} and ryanodine receptors are normally detected in cardiac myocytes. Testosterone did not change the frequency of these oscillations but increased their amplitude, possibly by increasing basal calcium levels. These oscillations may have an important regulatory function in the cardiac cells (48–50). The observation that intracellular Ca^{2+} oscillations induced by testosterone are seen only when there is a source of extracellular Ca^{2+} indicates that these oscillations are related to Ca^{2+} influx from the extracellular space. Ryanodine completely blocked the Ca^{2+} oscillations observed in testosterone-treated cardiac myocytes maintained in Ca^{2+}-containing medium. Because ryanodine inhibits the oscillatory behavior, the source of this phenomenon must rely both on Ca^{2+} influx across the plasma membrane and on the process of calcium-induced calcium release. Consistently, acute inhibition of L-type calcium channels also blocked Ca^{2+} oscillations but did not alter the slow release of Ca^{2+} from internal stores triggered by testosterone. This effect of L-type calcium channel blockers was different from that reported by others (34, 36), in which testosterone inhibits the dihydropyridine-sensitive calcium influx. These differences may rely on different properties of alternatively spliced generated isoforms of L-type Ca^{2+} channels between smooth and cardiac muscle cells (51).

The question arises as to the origin of the Ca^{2+} signal when there is no extracellular Ca^{2+}. Treatment with the Ca^{2+}-chelator BAPTA-AM completely abolished this increase in the absence of external Ca^{2+}, indicating that Ca^{2+} during this signal must be released from internal stores. Ryanodine-sensitive stores did not participate in the testosterone-induced slow Ca^{2+} release, and depletion of thapsigargin-sensitive Ca^{2+} stores from sarcoplasmic reticulum was in agreement with a role of intracellular Ca^{2+} in testosterone

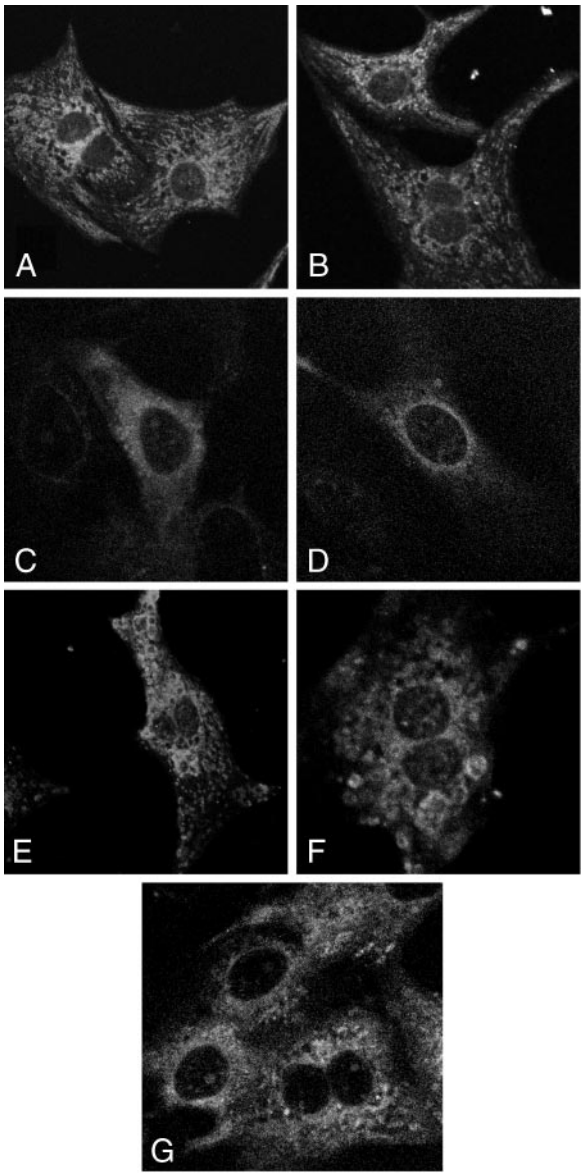

FIG. 5. Testosterone stimulates a slow intracellular androgen receptor translocation in cultured cardiac myocytes. Cells were stimulated with 100 nM testosterone for 0 min (A, control), 5 min (B), 1 h (C), or 2 h (D). Cells were pretreated with cyproterone and stimulated without (E) or with (F) testosterone for 2 h. Cells were stimulated with T-BSA for 2 h (G). After stimulation, the cardiac myocytes were fixed, incubated with an antibody against the intracellular androgen receptor, revealed with antirabbit IgG-FITC, and analyzed by confocal microscopy as described in *Materials and Methods*. Data are representative of three independent experiments.

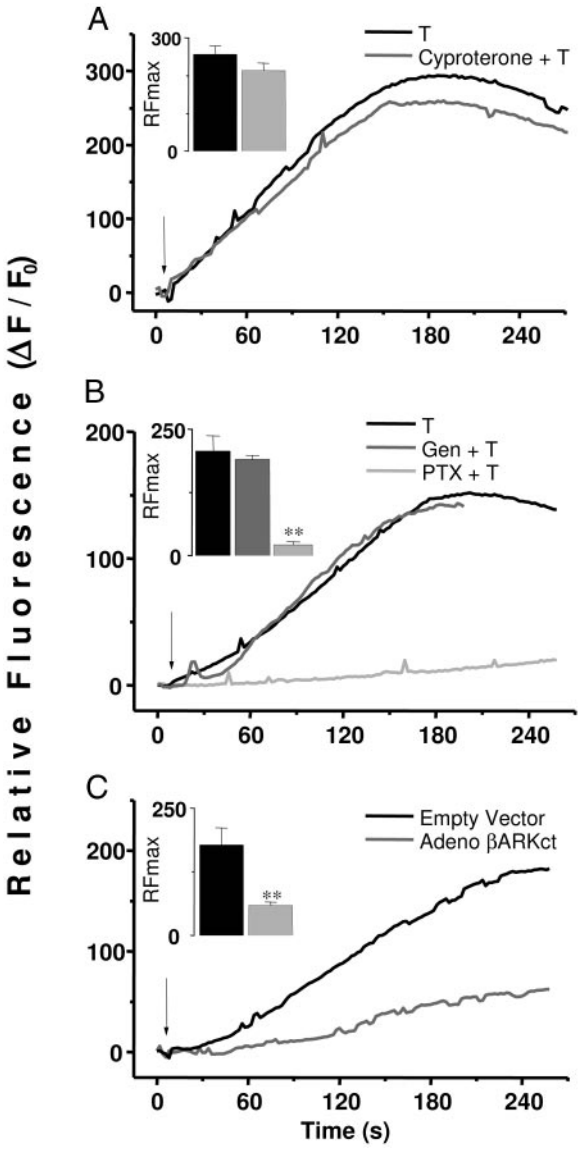

FIG. 6. Testosterone (T) increases intracellular Ca^{2+} levels by a mechanism involving a membrane androgen receptor, G protein, and $\beta\gamma$-dimmers in cultured cardiac myocytes. Cells were preloaded with fluo3-AM and maintained in a Ca^{2+}-free medium at the moment of stimulation. A, Cardiac myocytes were stimulated with testosterone (100 nM, *black line*) or pretreated with cyproterone (1 μM for 30 min, *gray line*) and then stimulated with testosterone. B, Cells were stimulated with testosterone (100 nM, *black line*) or pretreated with genistein (Gen, 50 μM for 30 min, *gray line*) or PTX (1 μg/ml for 6 h, *light gray line*) and then stimulated with testosterone. Genistein did not modify testosterone-induced Ca^{2+} signal, whereas PTX completely abolished this response. C, Cells were transduced with Ad-βARKct (a G$\beta\gamma$ signaling peptide inhibitor, MOI = 300 for 24 h) or Ad-EV (control, MOI = 300 for 24 h) and stimulated with testosterone (100 nM). The *inset graphics* correspond to the statistical analysis of RF max from the respective experiments performed with inhibitors and controls with testosterone. Data in *insets* are mean ± SE of triplicates from three independent experiments. **, $P < 0.01$ *vs.* controls.

action. We and others have described that testosterone-induced Ca^{2+} release from IP_3-sensitive stores in skeletal muscle cells and osteoblasts (14, 22). Our results using a PLC inhibitor and an IP_3 receptor blocker indicated that testosterone acted with a similar mechanism in cultured cardiac myocytes. The possibility of unspecific effects of U-73122 was discarded using the inactive isomer U-73343 as control. The time courses of both IP_3 mass and Ca^{2+} level increases support an IP_3-dependent Ca^{2+} release by testosterone. The experiments with specific inhibitors (BAPTA-AM, ryanodine, thapsigargin, U-73122, and xestospongin C) in Ca^{2+}-free medium are consistent with the sarcoplasmic reticulum being the source.

In many cell types, the actions of androgens involve bind-

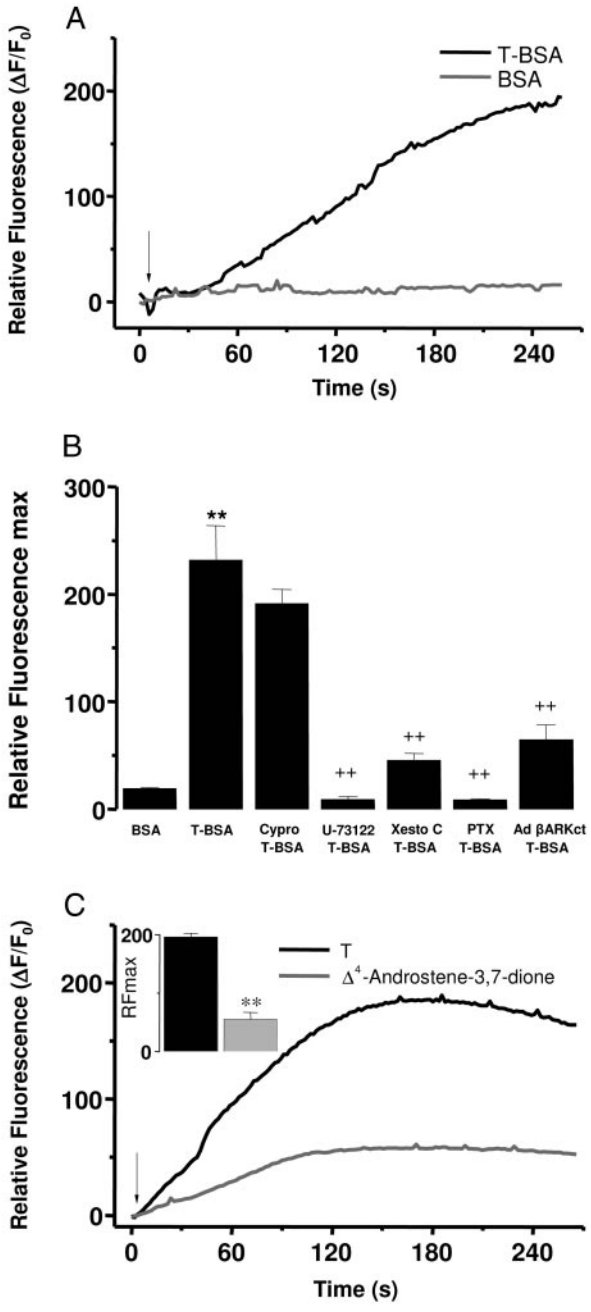

FIG. 7. Testosterone covalently bound to albumin effect on intracellular Ca^{2+} levels in cultured rat cardiac myocytes. Cells were preloaded with fluo3-AM and maintained in a Ca^{2+}-free medium at the moment of stimulation. A, Cells were stimulated with BSA (control, 100 nM, *gray line*) or T-BSA (100 nM, *black line*). B, Cells were pretreated with cyproterone (1 μM for 30 min), U-73122 (50 μM for 30 min), or xestospongin C (10 μM for 30 min), PTX (1 μg/ml for 6 h) or transduced with Ad-βARKct (MOI = 300 for 24 h). Cells were then stimulated with BSA (100 nM) or T-BSA (100 nM) as indicated. Values are expressed as mean ± SE of at least three independent experiments. **, $P < 0.01$ *vs.* BSA; $^{++}$, $P < 0.01$ *vs.* T-BSA. C, Cells were stimulated with testosterone (T, 100 nM, *black line*) or Δ^4-androstene-3,7-dione (100 nM, *gray line*), a metabolic precursor with less androgenic activity. The *inset graphic* is the statistical analysis of maximum relative fluorescence levels of cells treated with testosterone (*black line*) or Δ^4-androstene-3,7-dione (*gray line*). Values are expressed as mean ± SE of duplicates from at least three independent experiments. **, $P < 0.01$ *vs.* testosterone.

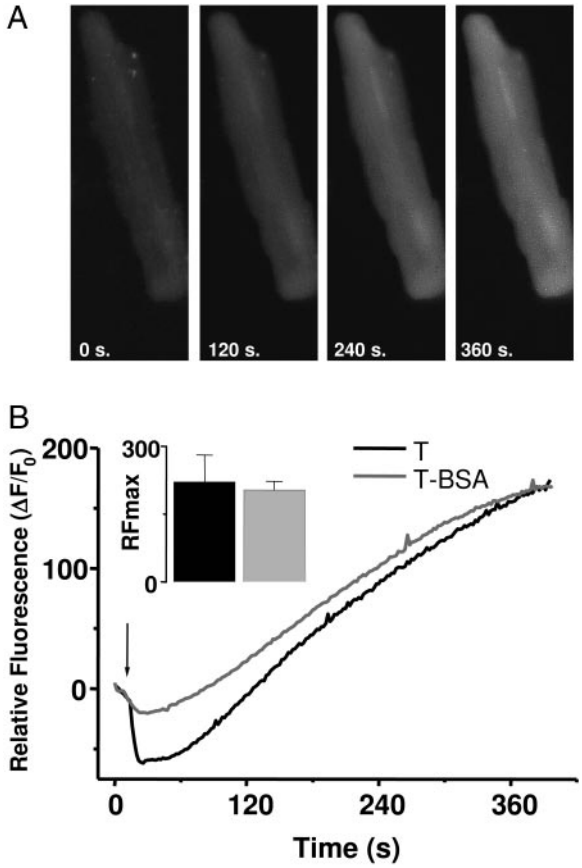

FIG. 8. Effect of testosterone (T) in adult rat cardiac myocytes. Cells were preloaded with fluo3-AM and maintained in Ca^{2+}-free medium at the moment of stimulation. A, Fluorescence images at the indicated times showing intracellular Ca^{2+} level changes in cardiac myocytes stimulated with testosterone 100 nM. B, $\Delta F/F_0$ calculated from the fluorescence images shown in A (*black line*) and cells treated with T-BSA (100 nM, *gray line*). The *inset graph* (B) corresponds to the statistical analysis of Rf max from experiments with testosterone and T-BSA. Values are expressed as mean ± SE of triplicates from three independent experiments. The time of stimulation is indicated by the *arrow*.

ing to intracellular receptors leading to genomic responses (52–54). However, there is now evidence to suggest that androgens also trigger rapid and nongenomic effects leading to the activation of several signal pathways (19, 55). In cardiac myocytes, we found that testosterone stimulates both intracellular androgen receptor translocation from the cytosol to perinuclear regions and induces intracellular Ca^{2+} transients. The former process is inhibited by cyproterone, whereas the latter is not. In addition, the kinetics of the changes in intracellular Ca^{2+} is sufficiently fast, so this process is unlikely to be mediated at the genomic level. The experiments with T-BSA strongly suggest that such short-term effects of the androgen may involve an action of the hormone primarily at the cell surface through a plasma membrane androgen receptor. Nongenomic responses originated by plasma membrane receptors have been described for steroids such as sex hormones, glucocorticoids, mineralocorticoids, neurosteroids, or vitamin D (55, 56). Others and we (14, 22, 57) have shown that the putative membrane androgen receptor is associated with G protein. Our results shown here

indicate that this G protein is sensitive to *pertussis* toxin and that the cascade also involves $\beta\gamma$-subunits.

Translocation of intracellular androgen receptor was inhibited by cyproterone, which has been previously reported to block genomic responses of androgens (42). The testosterone-induced Ca^{2+} signal was detected within 1–7 min and was not affected by cyproterone. Covalent linkage of testosterone to a large protein such as albumin impedes this hormone to cross the plasma membrane acting only at the plasma membrane level (18, 21, 58). Our results showed that T-BSA but not BSA triggered Ca^{2+} transients, suggesting the participation of a plasma membrane androgen receptor instead of an intracellular androgen receptor in the short-term effect of this hormone in cultured cardiac myocytes. To exclude any unspecific effect, additional experiments with Δ^4-androstene-3,7-dione, a metabolic precursor with reduced androgenic activity, validated the results. However, it cannot completely rule out the possibility that testosterone may activate an unrelated membrane receptor coupled to this pathway.

To check the effect of testosterone in differentiated cells, we used adult rat cardiac myocytes. The increase on intracellular calcium levels persisted, but the kinetics of the signal was slower. The persistence of the signal suggests that this nongenomic effect is a conserved mechanism of androgen action during development.

Cardiac hypertrophy is a leading predictor of progressive heart disease that often leads to heart failure and a loss of cardiac contractile performance associated with profound alterations in intracellular calcium handling. Different hormones and growth factors stimulate cardiac myocyte hypertrophy through Ca^{2+}-dependent signaling pathways (59). Calmodulin-activated phosphatase calcineurin, activated by increases in calcium, mediates the hypertrophic response through its downstream nuclear factor of activated T cells (60). Our results represent the first link between Ca^{2+} and AAS in cardiac myocytes, suggesting that this nongenomic effect of AAS can contribute to the documented androgen receptor mediated cardiotoxicity observed in AAS abuse. However, further work related with Ca^{2+} signaling pathways, such as calcineurin/nuclear factor of activated T cells, will be necessary to clarify the role of Ca^{2+} transients in the hypertrophic effects of androgens in the heart.

In conclusion, our results indicate that in rat neonatal cardiac myocytes, testosterone increases intracellular Ca^{2+} levels by a nongenomic mechanism, which involves a membrane androgen receptor, a PTX-sensitive G protein, PLC, IP_3, and IP_3R as signaling pathway.

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All authors have nothing to declare.

References

1. **Smeets L, Legros JJ** 2004 The heart and androgens. Ann Endocrinol (Paris) 65:163–170
2. **Evans NA** 2004 Current concepts in anabolic-androgenic steroids. Am J Sports Med 32:534–542
3. **Sullivan ML, Martinez CM, Gennis P, Gallagher EJ** 1998 The cardiac toxicity of anabolic steroids. Prog Cardiovasc Dis 41:1–15
4. **Freed DL, Banks AJ, Longson D, Burley DM** 1975 Anabolic steroids in athelics: crossover double-blind trial on weightlifters. Br Med J 2:471–473
5. **Lenders JW, Demacker PN, Vos JA, Jansen PL, Hoitsma AJ, van't LA, Thien T** 1988 Deleterious effects of anabolic steroids on serum lipoproteins, blood pressure, and liver function in amateur body builders. Int J Sports Med 9:19–23
6. **De Piccoli B, Giada F, Benettin A, Sartori F, Piccolo E** 1991 Anabolic steroid use in body builders: an echocardiographic study of left ventricle morphology and function. Int J Sports Med 12:408–412
7. **Pearson AC, Schiff M, Mrosek D, Labovitz AJ, Williams GA** 1986 Left ventricular diastolic function in weight lifters. Am J Cardiol 58:1254–1259
8. **Urhausen A, Holpes R, Kindermann W** 1989 One- and two-dimensional echocardiography in bodybuilders using anabolic steroids. Eur J Appl Physiol Occup Physiol 58:633–640
9. **Nieminen MS, Ramo MP, Viitasalo M, Heikkila P, Karjalainen J, Mantysaari M, Heikkila J** 1996 Serious cardiovascular side effects of large doses of anabolic steroids in weight lifters. Eur Heart J 17:1576–1583
10. **Thiblin I, Lindquist O, Rajs J** 2000 Cause and manner of death among users of anabolic androgenic steroids. J Forensic Sci 45:16–23
11. **English KM, Jones RD, Jones TH, Morice AH, Channer KS** 2002 Testosterone acts as a coronary vasodilator by a calcium antagonistic action. J Endocrinol Invest 25:455–458
12. **Callies F, Stromer H, Schwinger RH, Bolck B, Hu K, Frantz S, Leupold A, Beer S, Allolio B, Bonz AW** 2003 Administration of testosterone is associated with a reduced susceptibility to myocardial ischemia. Endocrinology 144: 4478–4483
13. **Er F, Michels G, Gassanov N, Rivero F, Hoppe UC** 2004 Testosterone induces cytoprotection by activating ATP-sensitive K+ channels in the cardiac mitochondrial inner membrane. Circulation 110:3100–3107
14. **Lieberherr M, Grosse B** 1994 Androgens increase intracellular calcium concentration and inositol 1,4,5-trisphosphate and diacylglycerol formation via a pertussis toxin-sensitive G-protein. J Biol Chem 269:7217–7223
15. **Benten WP, Lieberherr M, Stamm O, Wrehlke C, Guo Z, Wunderlich F** 1999 Testosterone signaling through internalizable surface receptors in androgen receptor-free macrophages. Mol Biol Cell 10:3113–3123
16. **Estrada M, Liberona JL, Miranda M, Jaimovich E** 2000 Aldosterone- and testosterone-mediated intracellular calcium response in skeletal muscle cell cultures. Am J Physiol Endocrinol Metab 279:E132–E139
17. **Wehling M, Christ M, Theisen K** 1992 Membrane receptors for aldosterone: a novel pathway for mineralocorticoid action. Am J Physiol 263:E974–E979
18. **Benten WP, Lieberherr M, Giese G, Wrehlke C, Stamm O, Sekeris CE, Mossmann H, Wunderlich F** 1999 Functional testosterone receptors in plasma membranes of T cells. FASEB J 13:123–133
19. **Wehling M** 1997 Specific, nongenomic actions of steroid hormones. Annu Rev Physiol 59:365–393
20. **Benten WP, Lieberherr M, Sekeris CE, Wunderlich F** 1997 Testosterone induces Ca2+ influx via nongenomic surface receptors in activated T cells. FEBS Lett 407:211–214
21. **Wunderlich F, Benten WP, Lieberherr M, Guo Z, Stamm O, Wrehlke C, Sekeris CE, Mossmann H** 2002 Testosterone signaling in T cells and macrophages. Steroids 67:535–538
22. **Estrada M, Espinosa A, Muller M, Jaimovich E** 2003 Testosterone stimulates intracellular calcium release and mitogen-activated protein kinases via a G protein-coupled receptor in skeletal muscle cells. Endocrinology 144:3586–3597
23. **Gerasimenko O, Gerasimenko J** 2004 New aspects of nuclear calcium signalling. J Cell Sci 117:3087–3094
24. **Ruizeveld de Winter JA, Trapman J, Vermey M, Mulder E, Zegers ND, van der Kwast TH** 1991 Androgen receptor expression in human tissues: an immunohistochemical study. J Histochem Cytochem 39:927–936
25. **Golden KL, Marsh JD, Jiang Y, Moulden J** 2004 Gonadectomy alters myosin heavy chain composition in isolated cardiac myocytes. Endocrine 24:137–140
26. **Golden KL, Marsh JD, Jiang Y** 2004 Testosterone regulates mRNA levels of calcium regulatory proteins in cardiac myocytes. Horm Metab Res 36:197–202
27. **Foncea R, Andersson M, Ketterman A, Blakesley V, Sapag-Hagar M, Sugden PH, LeRoith D, Lavandero S** 1997 Insulin-like growth factor-I rapidly activates multiple signal transduction pathways in cultured rat cardiac myocytes. J Biol Chem 272:19115–19124

28. **Shah AS, White DC, Emani S, Kypson AP, Lilly RE, Wilson K, Glower DD, Lefkowitz RJ, Koch WJ** 2001 *In vivo* ventricular gene delivery of a β-adrenergic receptor kinase inhibitor to the failing heart reverses cardiac dysfunction. Circulation 103:1311–1316
29. **Koch WJ, Hawes BE, Inglese J, Luttrell LM, Lefkowitz RJ** 1994 Cellular expression of the carboxyl terminus of a G protein-coupled receptor kinase attenuates G $\beta\gamma$-mediated signaling. J Biol Chem 269:6193–6197
30. **Minta A, Kao JP, Tsien RY** 1989 Fluorescent indicators for cytosolic calcium based on rhodamine and fluorescein chromophores. J Biol Chem 264:8171–8178
31. **Castleman K** 1989 Digital image processing. Englewood Cliffs, NJ: Prentice-Hall
32. **Liberona JL, Powell JA, Shenoi S, Petherbridge L, Caviedes R, Jaimovich E** 1998 Differences in both inositol 1,4,5-trisphosphate mass and inositol 1,4,5-trisphosphate receptors between normal and dystrophic skeletal muscle cell lines. Muscle Nerve 21:902–909
33. **Lowry OH, Rosebrough NJ, Farr AL, Randall RJ** 1951 Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275
34. **Perusquia M, Navarrete E, Jasso-Kamel J, Montano LM** 2005 Androgens induce relaxation of contractile activity in pregnant human myometrium at term: a nongenomic action on L-type calcium channels. Biol Reprod 73:214–221
35. **Jones RD, English KM, Jones TH, Channer KS** 2004 Testosterone-induced coronary vasodilatation occurs via a non-genomic mechanism: evidence of a direct calcium antagonism action. Clin Sci (Lond) 107:149–158
36. **Scragg JL, Jones RD, Channer KS, Jones TH, Peers C** 2004 Testosterone is a potent inhibitor of L-type Ca(2+) channels. Biochem Biophys Res Commun 318:503–506
37. **Wang JP** 1996 U-73122, an aminosteroid phospholipase C inhibitor, may also block Ca^{2+} influx through phospholipase C-independent mechanism in neutrophil activation. Naunyn Schmiedebergs Arch Pharmacol 353:599–605
38. **Muto Y, Nagao T, Urushidani T** 1997 The putative phospholipase C inhibitor U73122 and its negative control, U73343, elicit unexpected effects on the rabbit parietal cell. J Pharmacol Exp Ther 282:1379–1388
39. **Walker EM, Bispham JR, Hill SJ** 1998 Nonselective effects of the putative phospholipase C inhibitor, U73122, on adenosine A1 receptor-mediated signal transduction events in Chinese hamster ovary cells. Biochem Pharmacol 56:1455–1462
40. **Cenni B, Picard D** 1999 Two compounds commonly used for phospholipase C inhibition activate the nuclear estrogen receptors. Biochem Biophys Res Commun 261:340–344
41. **Simental JA, Sar M, Lane MV, French FS, Wilson EM** 1991 Transcriptional activation and nuclear targeting signals of the human androgen receptor. J Biol Chem 266:510–518
42. **Kuil CW, Mulder E** 1996 Deoxyribonucleic acid-binding ability of androgen receptors in whole cells: implications for the actions of androgens and antiandrogens. Endocrinology 137:1870–1877
43. **Blackmore PF, Neulen J, Lattanzio F, Beebe SJ** 1991 Cell surface-binding sites for progesterone mediate calcium uptake in human sperm. J Biol Chem 266:18655–18659
44. **Guo Z, Benten WP, Krucken J, Wunderlich F** 2002 Nongenomic testosterone calcium signaling. Genotropic actions in androgen receptor-free macrophages. J Biol Chem 277:29600–29607
45. **Wyckoff MH, Chambliss KL, Mineo C, Yuhanna IS, Mendelsohn ME, Mumby SM, Shaul PW** 2001 Plasma membrane estrogen receptors are coupled to endothelial nitric-oxide synthase through Gα(i). J Biol Chem 276:27071–27076
46. **Peng SH, Segura J, Farre M, Gonzalez JC, de la Torre X** 2002 Plasma and urinary markers of oral testosterone undecanoate misuse. Steroids 67:39–50
47. **Bhasin S, Woodhouse L, Casaburi R, Singh AB, Bhasin D, Berman N, Chen X, Yarasheski KE, Magliano L, Dzekov C, Dzekov J, Bross R, Phillips J, Sinha-Hikim I, Shen R, Storer TW** 2001 Testosterone dose-response relationships in healthy young men. Am J Physiol Endocrinol Metab 281:E1172–E1181
48. **Crabtree GR** 2001 Calcium, calcineurin, and the control of transcription. J Biol Chem 276:2313–2316
49. **Stull JT** 2001 Ca^{2+}-dependent cell signaling through calmodulin-activated protein phosphatase and protein kinases minireview series. J Biol Chem 276:2311–2312
50. **Vega RB, Bassel-Duby R, Olson EN** 2003 Control of cardiac growth and function by calcineurin signaling. J Biol Chem 278:36981–36984
51. **Liao P, Yu D, Lu S, Tang Z, Liang MC, Zeng S, Lin W, Soong TW** 2004 Smooth muscle-selective alternatively spliced exon generates functional variation in Cav1.2 calcium channels. J Biol Chem 279:50329–50335
52. **Quigley CA, De Bellis A, Marschke KB, el Awady MK, Wilson EM, French FS** 1995 Androgen receptor defects: historical, clinical, and molecular perspectives. Endocr Rev 16:271–321
53. **Zhou ZX, Wong CI, Sar M, Wilson EM** 1994 The androgen receptor: an overview. Recent Prog Horm Res 49:249–274
54. **Ikeda Y, Aihara K, Sato T, Akaike M, Yoshizumi M, Suzaki Y, Izawa Y, Fujimura M, Hashizume S, Kato M, Yagi S, Tamaki T, Kawano H, Matsumoto T, Azuma H, Kato S, Matsumoto T** 2005 Androgen receptor gene knockout male mice exhibit impaired cardiac growth and exacerbation of angiotensin II-induced cardiac fibrosis. J Biol Chem 280:29661–29666
55. **Wehling M** 1995 Looking beyond the dogma of genomic steroid action: insights and facts of the 1990s. J Mol Med 73:439–447
56. **Suzuki T, Nakamura Y, Moriya T, Sasano H** 2003 Effects of steroid hormones on vascular functions. Microsc Res Tech 60:76–84
57. **Zagar Y, Chaumaz G, Lieberherr M** 2004 Signaling cross-talk from Gβ4 subunit to Elk-1 in the rapid action of androgens. J Biol Chem 279:2403–2413
58. **Armen TA, Gay CV** 2000 Simultaneous detection and functional response of testosterone and estradiol receptors in osteoblast plasma membranes. J Cell Biochem 79:620–627
59. **Frey N, McKinsey TA, Olson EN** 2000 Decoding calcium signals involved in cardiac growth and function. Nat Med 6:1221–1227
60. **Wilkins BJ, Molkentin JD** 2004 Calcium-calcineurin signaling in the regulation of cardiac hypertrophy. Biochem Biophys Res Commun 322:1178–1191