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Androgens Induce Increases in Intracellular Calcium Via a G Protein-Coupled Receptor in  
LNCap Prostate Cancer Cells

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## ABSTRACT

The receptor mechanism of testosterone-induced nongenomic  $\text{Ca}^{2+}$  signaling in prostate cancer cells is poorly understood. In this study, we investigated androgens-induced intracellular  $\text{Ca}^{2+}$  increase in LNCaP human prostate cancer cells by using fura-2 as a  $\text{Ca}^{2+}$  probe. 5 $\alpha$ -dihydrotestosterone (DHT) produced fast and transient increases in intracellular  $\text{Ca}^{2+}$  in LNCaP cells in a concentration-dependent manner. These effects were abolished by extracellular  $\text{Ca}^{2+}$  removal or pretreatment with L-type  $\text{Ca}^{2+}$  channel inhibitors (Nifedipine, Verapamil and Diltiazem). Pretreatment with endoplasmic reticulum ryanodine receptor blocker (procaine) or phospholipase C inhibitor (neomycin sulfate) did not alter DHT induced  $\text{Ca}^{2+}$  influx.  $\text{Ca}^{2+}$  increase could also be induced by impermeable testosterone conjugated to BSA. Neither antagonist of intracellular androgen receptors (CPA) nor protein synthesis inhibitor (cycloheximide) affected this fast  $\text{Ca}^{2+}$  influx. Furthermore, the effect of DHT was abolished in cells incubated with G protein inhibitor (pertussis toxin) and a nonhydrolyzable analog of GTP (GDP $\beta$ S), but not in cells incubated with the tyrosine kinase inhibitor (genistein). The results indicate that androgens induced an L-type calcium channel dependent intracellular calcium increase in LNCaP prostate cancer cells. The rapid responses triggered by DHT did not appear to be mediated through classic intracellular androgen receptors or c-Src kinase-AR complex or sex hormone-binding globulin (SHBG), but through a G protein-coupled receptor in LNCaP Prostate Cancer Cells. These results might provide a new explanation for progression of prostate cancer.

**Key Words:** 5 $\alpha$ -dihydrotestosterone;  $\text{Ca}^{2+}$ ; prostate cancer cells; G Protein-Coupled Receptor

Appropriate binding of androgen to its receptor is necessary for the development and progression of prostate cancer. Testosterone, the principal steroidal androgen, and its metabolite 5-dihydrotestosterone (DHT), are thought to mediate their biological effects through binding to the intracellular androgen receptor (AR). AR, in common with other members of the nuclear receptor superfamily, functions as a ligand-inducible transcription factor. Binding of testosterone or DHT to AR induces receptor dimerization, facilitating the ability of AR to bind to its cognate response element and recruit coregulators to promote the expression of target genes.

In addition, the effect of androgens, as was reported in a number of studies, was very rapid, occurring in minutes, a time lag noncompatible with the classical scheme of a nuclear receptor action (Wehling, 1997). These findings led to the identification of nongenomic actions of testosterone through membrane androgen receptors (mAR) on cell surfaces. The nongenomic effects of testosterone include calcium mobilization, secretion and cytoskeleton modifications (Kampa et al, 2002), regulated by the activation of signaling molecules. Androgens can induce rapid calcium fluxes in a variety of cell types, including human prostate cancer cells (Steinsapir et al, 1991), rat heart myocytes (Koenig et al, 1989), male (but not female) rat osteoblasts (Lieberherr et al, 1989), and mouse T cells, in which the presence of a functional classical androgen receptor could not be demonstrated (Benten et al, 1999). Also, human granulosa cells were shown to make a calcium response to androstenedione but not to testosterone (Machelon et al, 1998). Furthermore, a rapid calcium influx was observed after adding high concentrations of androgens to freshly isolated immature rat Sertoli cells (Gorczyńska and Handelsman, 1995).

60 The mechanism of the nongenomic effects of androgen varies with cell types. In murine T-cells, for example, mAR mediates ligand induced  $\text{Ca}^{2+}$  influx through non-voltage-gated,  $\text{Ni}^{2+}$ -blockable  $\text{Ca}^{2+}$  channels (Benten et al, 1999). In rat osteoblasts, testosterone induces both extracellular  $\text{Ca}^{2+}$  influx via voltage-gated  $\text{Ca}^{2+}$  channels and  $\text{Ca}^{2+}$  release from intracellular stores through G-protein-coupled receptors activating phospholipase C via a pertussis  
65 toxin-sensitive G-protein (Lieberherr et al, 1989). Murine macrophages of the cell line IC-21 respond to testosterone with predominantly intracellular  $\text{Ca}^{2+}$  mobilization mediated through G protein-coupled receptors (GPCR) for testosterone (Wunderlich et al, 2002). However, the receptor mechanism of testosterone-induced nongenomic  $\text{Ca}^{2+}$  signaling in prostate cancer cells is poorly understood.

70 In the present study, we investigated the mechanism of the effect of DHT on  $[\text{Ca}^{2+}]_i$  in LNCaP human prostate cancer cells. By using fura-2 as a  $\text{Ca}^{2+}$  probe, we found that androgen caused a significant increase in  $[\text{Ca}^{2+}]_i$ . The concentration-response relationship has been established, and the sources and the mechanisms of the  $[\text{Ca}^{2+}]_i$  increase have been explored. We report that androgens induce calcium influx via a GPCR in LNCaP prostate cancer cells.

## 75 MATERIALS AND METHODS

*Cell Culture*

LNCaP cell line was purchased from the American Type Culture Collection (ATCC). Cells were grown in normal RPMI 1640 medium without phenol red supplemented with 10% heat-inactivated fetal bovine serum (FCS) at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>.

80 Cells were subcultured once a week and incubated in serum-free medium for 24 h before any experiment.

*Solutions*

Krebs-HEPES (pH 7.4) contained 125mM NaCl, 5.6mM KCl, 2.2mM CaCl<sub>2</sub>, 1.2mM MgSO<sub>4</sub>, 1.2mM NaH<sub>2</sub>PO<sub>4</sub>, 10mM HEPES, and 10mM Glucose. Ca<sup>2+</sup>-free Krebs-HEPES contained 85 added Ca<sup>2+</sup> plus 1 mM EGTA to chelate residual Ca<sup>2+</sup>. The experimental solution contained less than 0.1% of solvent (dimethyl sulfoxide or ethanol) which did not affect [Ca<sup>2+</sup>]<sub>i</sub> (n=3).

*Measurement of Intracellular Free Ca<sup>2+</sup> Concentration ([Ca<sup>2+</sup>]<sub>i</sub>)*

[Ca<sup>2+</sup>]<sub>i</sub> was measured as described previously (Huang and Jan, 2001). Cells (10<sup>6</sup>/ml) were planted on a cover slip and loaded with the ester form of fura-2, fura-2/AM (10 μM) for 30 min at 37°C in 90 Krebs-HEPES, and washed with Krebs-HEPES before use. Fura-2 Fluorescence was imaged on an Olympus IX-70 inverted microscope with a 75-W xenon arc lamp equipped with a rotating filter wheel (Lambda 10-2; Sutter Instruments) and a cooled CCD camera, CoolSNAP-HQ, controlled by MetaFluor (Roper Scientific, Trenton, NJ). The excitation signals at 340 and 380 nm and emission signal at 510 nm were recorded at 1 sec intervals. The Fura-2 signal was converted to [Ca<sup>2+</sup>]<sub>i</sub> using 95 an in vitro calibration method. The relationship between [Ca<sup>2+</sup>]<sub>i</sub> and the ratio R of fluorescence intensity ratio at 340/380 nm excitation is:

$$[Ca^{2+}]_i = K_d \beta [(R - R_{min}) / (R_{max} - R)]$$

$R_{min}$  and  $R_{max}$  refer to ratio values at zero  $[Ca^{2+}]$  and saturating  $[Ca^{2+}]$ , respectively. Data of Maximum and minimum fluorescence values were obtained by adding 0.1% Triton X-100 and  
100 20 mM EGTA sequentially at the end of each experiment.  $\beta$  is the emission intensity ratio at zero and saturating  $[Ca^{2+}]$  at 380 nm excitation;  $K_d$  is the dissociation constant of Fura-2 for  $Ca^{2+}$ ; a value of 155 nM was previously determined (Grynkiewicz et al, 1985).

#### *Chemical Reagents*

Testosterone, Verapamil, Diltiazem, Nifedipine, Neomycin sulfate, Procaine, Cycloheximide,  
105 Cyproterone acetate (CPA), EGTA, testosterone-BSA (29 mol steroid/mol BSA), and Genistein were obtained from the Sigma Chemical Company (St Louis, Mo). Pertussis toxin, GDP $\beta$ s and saponin were obtained from Calbiochem (San Diego, California, United States). Dihydrotestosterone was a gift from Professor Jung (Chcerite Medical school, Germany). All compounds were dissolved in ethanol. Fura-2/AM (from Molecular Probes, Leiden, The  
110 Netherlands) was dissolved in DMSO.

#### *Statistical Analyses*

Data were reported as the mean  $\pm$  SEM (n=40-60). Statistical analysis was performed with software (SPSS 11.5, SPSS Inc., Chicago, Illinois) and the one way ANOVA using LSD or SNK method was used to evaluate the possible differences across groups. Significance was  
115 accepted when  $P < 0.05$ .

## RESULTS

### *Effect of Androgens on $[Ca^{2+}]_i$*

In normal Krebs-HEPES, DHT (1-1000 nM) increased  $[Ca^{2+}]_i$  concentration-dependently (Fig. 1A, B). The basal  $[Ca^{2+}]_i$  was about 28-30 nM. At a concentration of 1 nM DHT had no effect. At a concentration of 10 nM, DHT induced a  $[Ca^{2+}]_i$  increase which reached a maximum value at the time point of 25 sec (n=40;  $P<0.05$ ). At a concentration of 100 nM, DHT induced a  $[Ca^{2+}]_i$  increase which reached a maximum value of  $193\pm 33$  nM (n=40;  $P<0.001$ ) after at least 3 min. The response induced by 1  $\mu$ M DHT was similar to that induced by 100 nM DTH. The effects of DHT (100 nm) on intracellular calcium in LNCaP cells are shown in Fig. 1C, which represents a sequence of fluorescence images acquired at the times indicated. In this experiment, a fast increase in the fluorescence of LNCaP cells preloaded with fura-2/AM after hormone exposition was observed.

### *Effect of Extracellular $Ca^{2+}$ Removal and L-Type Calcium Channel Inhibitors on the Androgen Responses*

The extra- or intracellular origin of the calcium involved in these signals was investigated using a variety of experimental conditions. First, cells were incubated in a virtually  $Ca^{2+}$  free medium (1mM EGTA) prior to androgen stimulation. Extracellular  $Ca^{2+}$  removal inhibited the DHT-induced  $[Ca^{2+}]_i$  increase (n=50;  $P<0.05$ ). The basal  $[Ca^{2+}]_i$  was  $9\pm 2$  nM. The concentration-response relationships of DHT-induced  $[Ca^{2+}]_i$  increases in the presence and absence of  $Ca^{2+}$  are shown in Figure 2A.

Furthermore, pretreatment with Nifedipine (5 mM), Verapamil (50  $\mu$ M) and Diltiazem (100  $\mu$ M), inhibitors of L-type voltage-gated calcium channels for 5min and addition of 1000nM

DHT did not increase  $[Ca^{2+}]_i$  (Figure 2A), indicating that androgen-induced calcium increases originated from an extracellular source through L-type voltage-gated calcium channels.

140 *Intracellular  $Ca^{2+}$  Stores are not Involved in Androgen Responses*

The contribution of the  $Ca^{2+}$  stores in the endoplasmic reticulum was examined. Procaine is an inhibitor of ryanodine receptor, which was shown to release endoplasmic reticulum  $Ca^{2+}$  in cells. Pretreatment with this drug did not modify the  $Ca^{2+}$  increase induced by DHT. Figure 2B shows that pretreatment of 50 mM Procaine for 3 min and addition of DHT induced an  
145 immediate increase in  $[Ca^{2+}]_i$  with a peak value of  $205 \pm 44$  nM ( $n=45$ ;  $P<0.001$ ).

Experiments were performed to examine whether androgens released  $Ca^{2+}$  via stimulating inositol 1,4,5-trisphosphate formation, by exploring the inhibitory effect of phospholipase C on androgens-induced  $[Ca^{2+}]_i$  increase. Figure 2B shows that pretreatment with 1 mM Neomycin sulfate, a phospholipase C inhibitor, for 5 min and addition of DTH (1000 nM) induced an  
150 immediate  $[Ca^{2+}]_i$  increase with a peak value of  $201 \pm 91$  nM indistinguishable from control shown in Figure 1A ( $n=55$ ,  $P<0.001$ ), indicating that androgen-induced calcium increase did not originate from intracellular calcium stores.

*Androgen Induced Intracellular Calcium is Mediated by a Nongenomic Mechanism*

The possibility that androgens induce  $[Ca^{2+}]_i$  increases in a receptor-dependent manner was  
155 examined. If the intracellular androgen receptor was responsible for the androgen-triggered calcium increase in LNCaP cells, this increase should be blocked by CPA, an antagonist of the intracellular androgen receptor. CPA has been shown to block genomic activation in a number of cell systems. However, the intracellular  $Ca^{2+}$  transient triggered by DHT ( $n=60$ ) was not affected by pretreating the cells with a high concentration (1  $\mu$ M) of CPA for 30 min (Fig 3A).

160 Furthermore, pretreatment with 10 $\mu$ M Cycloheximide, an inhibitor in protein synthesis, for 3 hr, did not affect intracellular Ca<sup>2+</sup> transient triggered by DHT, suggesting that the effect of the hormone was mediated by a nongenomic mechanism.

Rapid testosterone effects (within the first minute) involving second messengers have been reported in other cell types, and nongenomic mechanisms of signal transduction have been  
165 proposed. Testosterone is an analog of DHT; it mimics the effects of DHT, suggesting that intracellular calcium increase is a common pathway for androgen steroid action in LNCaP prostate cancer cells. To evaluate whether the effect of the hormone was mediated by extracellular membrane receptors, we tested the effect of testosterone covalently bound to albumin (T-BSA). This compound does not cross the plasma membrane, nor has it been reported  
170 to act on intracellular androgen receptors. T-BSA produced intracellular calcium increases in LNCaP cells. BSA (0.1%) did not produce any change in the intracellular Ca<sup>2+</sup> (Fig. 3B).

*Androgen Stimulates Intracellular Calcium Release via a GPCR in LNCaP Prostate Cancer Cells*

To determine the early events involved in the Ca<sup>2+</sup> signal produced by DHT, LNCaP cells were  
175 incubated for 20 min with 100  $\mu$ M genistein, a tyrosine kinase inhibitor, prior to hormone stimulation. This inhibitor did not modify the DHT-induced intracellular Ca<sup>2+</sup> increases (Fig. 3C). On the other hand, a role for a G protein in this effect was evaluated. LNCaP cells were permeabilized for 5 min with saponin in the presence of 500  $\mu$ M GDP $\beta$ S, a nonhydrolyzable analog of GTP. Permeabilization did not modify DHT-induced responses, while GDP $\beta$ S  
180 suppressed the Ca<sup>2+</sup> increases induced by the hormone. Furthermore, cells were incubated with 100 ng/ml PTX (Pertussis toxin) before DHT stimulation. Figure 3C shows that PTX inhibited

the  $\text{Ca}^{2+}$  signals produced by the hormone. These results suggested that androgen action required a PTX sensitive G protein to produce  $\text{Ca}^{2+}$  increases in LNCaP prostate cancer cells.

## DISCUSSION

185 This study examined the mechanism of androgens-induced rapid calcium increase in LNCaP human prostate cancer cells. The results suggest that DHT and testosterone induced rapid  $[Ca^{2+}]_i$  increase at 10nM. Since the plasma concentration of testosterone was between 12-27 nM, our results suggest that the clinical plasma level of testosterone may alter  $Ca^{2+}$  signaling in patients' prostate cancer cells. We show here suggestive evidence of a G protein-linked membrane  
190 receptor activated by androgens in LNCaP prostate cancer cells. Activation of this receptor resulted in intracellular calcium transients, which appears to depend on L-type calcium channel on cell membrane. Furthermore, CPA, an antagonist of intracellular androgen receptors, did not inhibit increases in androgen-induced calcium influx. Moreover, testosterone bound to a large protein molecule (T-BSA) mimics these effects. These results suggest that the rapid responses  
195 triggered by DHT were not due to activation of the classical intracellular androgen receptor in LNCaP prostate cancer cells, but through a G protein-linked membrane receptor.

Testosterone has been reported to induce intracellular calcium increases in rat osteoblasts, myotubes, mice splenic T cells, macrophages and human prostate cancer cells. Consistent with the previous study (Steinsapir et al, 1991), we found that DHT induced  $[Ca^{2+}]_i$  increases in  
200 LNCaP cells by using fura-2 as a  $Ca^{2+}$  probe. More over, we have given a series of fluorescence ratio images, in pseudocolor, from a LNCaP cell preloaded with Fura-2/AM dye. The sequence showed a fast and transient fluorescence increase after testosterone addition more intuitively. It is reported that sustained elevation of intracellular  $Ca^{2+}$  with  $Ca^{2+}$  ionophores or inhibitors of  $Ca^{2+}$ -ATPase have been found to reduce AR expression (Gong et al, 1995) and  
205 promote apoptosis in prostate cancer cells (Tombal et al, 2000). The effect of physiological

exposure to androgen on  $\text{Ca}^{2+}$ -mediated functions remains to be investigated.

Another question is how DHT induces  $\text{Ca}^{2+}$  increase. The source of calcium increase varies with cell type. In murine T-cells, for example, mAR mediates ligand induced  $\text{Ca}^{2+}$  influx through non-voltage-gated,  $\text{Ni}^{2+}$ -blockable  $\text{Ca}^{2+}$  channels. In rat osteoblasts, testosterone induces both the influx of extracellular  $\text{Ca}^{2+}$  via voltage-gated  $\text{Ca}^{2+}$  channels and  $\text{Ca}^{2+}$  release from intracellular stores through G-protein-coupled receptors activating phospholipase C via a pertussis toxin-sensitive G-protein. Murine macrophages of the cell line IC-21 respond to testosterone with predominantly intracellular  $\text{Ca}^{2+}$  mobilization mediated through GPCR for testosterone. Our results suggest that the effects of androgen on LNCaP cells were abolished by extracellular  $\text{Ca}^{2+}$  removal or pretreatment with L-type  $\text{Ca}^{2+}$  channel inhibitors (Nifedipine, Verapamil and Diltiazem). These results are consistent with the finding of the previous study that androgens may directly cause  $\text{Ca}^{2+}$  entry through L-type calcium channel in LNCaP cells (Steinsapir et al, 1991). Furthermore, we found that pretreatment with endoplasmic reticulum ryanodine receptor blocker (procaine) or phospholipase C inhibitor (neomycin sulfate) did not alter the DHT induced  $\text{Ca}^{2+}$  influx. These results support the possibility that androgens may cause  $\text{Ca}^{2+}$  increase in a manner dissociated from  $\text{Ca}^{2+}$  store depletion and intracellular  $\text{Ca}^{2+}$  stores activation.

It has not yet been determined whether the nongenomic effects of androgen are mediated through a novel membrane androgen receptor or through a c-Src kinase-AR complex. AR has been found to interact with the intracellular tyrosine kinase c-Src, triggering c-Src activation. The tyrosine kinase activity of c-Src is autoinhibited by the interaction between the tyrosine kinase domain and the Src homology 2 (SH2) and Src homology 3 (SH3) domains. In response

to DHT or the synthetic androgen R1881, AR interacts with the SH3 domain of c-Src. The association of AR with c-Src results in stimulation of c-Src kinase activity within 1 min in the AR-positive LNCaP prostate cancer cell line in response to 10 nM R1881. The rapidity of c-Src kinase activation suggests that R1881 stimulates the c-Src pathway through a nongenomic mechanism (Heinlein and Chang, 2002). In the present study, we found that membrane-impermeant testosterone conjugate (T-BSA) induced similar effects on calcium compared with the free hormone, excluding the possibility that rapid  $\text{Ca}^{2+}$  increase effects of androgen on LNCaP cells were mediated through intracellular c-Src kinase-AR complex. Furthermore, we found that androgen-induced calcium influx was not blunted by CPA, an antagonist of the intracellular androgen receptor, suggesting that these rapid effects are not mediated by the actions of classic intracellular AR, but by a trans-membrane androgen receptor.

The nongenomic action of androgen has been reported to be mediated by activation of tyrosine kinase receptor as well as GPCR. It was reported that an androgen analog, antiandrogen hydroxyflutamide, exerted a non-AR-mediated and nongenomic action in AR-negative prostate cancer cells through EGF receptor, a tyrosine kinase receptor (Lee et al, 2002). Yet, in the present study, treatment of LNCaP cells with genistein, a tyrosine kinase inhibitor, did not modify the intracellular  $\text{Ca}^{2+}$  increase induced by testosterone. On the other hand, several lines of evidence demonstrate that androgens can activate PTX-sensitive G proteins. To determine whether testosterone may interact with a GPCR in LNCaP cells, we evaluated  $\text{Ca}^{2+}$  increase in LNCaP cells treated with  $\text{GDP}\beta\text{S}$  and PTX. The fact that G protein inhibitors blocked fast effects of testosterone reinforced the idea of a membrane receptor for this hormone. Androgen binding by SHBG could also act membrane SHBG receptor (SHBG-R), which has also been

250 suggested to be coupled to G proteins and stimulate cAMP and PKA. However, the SHBG receptor is associated with G<sub>s</sub> containing G protein complexes (Rosner et al, 1999). We found that the intracellular Ca<sup>2+</sup> increase in LNCaP cells was sensitive to PTX, which does not inhibit the G<sub>s</sub> subfamily, suggesting that GPCR mediating intracellular calcium elevation in LNCaP cells is distinct from the SHBG receptor.

255 The existence of a novel membrane androgen receptor has been postulated by a number of authors based on the detection of specific androgen binding to plasma membranes in different cell types. Unfortunately this putative membrane receptor has not yet been further purified or cloned, preventing a definitive characterization. A human membrane receptor for progesterone has been cloned and a heteromeric membrane receptor for anabolic steroids has recently been  
260 isolated (Gerdes et al, 1998). The identification of distinct membrane receptors for other steroid hormones suggests that a novel membrane receptor for androgens may also exist. Moreover, a number of receptors are known to be coupled to more than one G subfamily (Gudermann et al, 1997). Therefore, it remains to be determined whether androgen action via GPCR occurs through a single receptor coupled to different G subfamilies, possibly in a tissue- specific  
265 manner, or through separate receptors each linked to specific G protein complexes.

The mechanism of the change in prostate cancer from being androgen-responsive to androgen-unresponsive is generally explained by clonal selection, adaptation, an alternative pathway of signal transduction and androgen receptor (AR) involvement. Since androgen action is mediated by AR, abnormalities in AR are believed to play an important role in the progression  
270 of prostate cancer (Suzuki et al, 2003). Our experiment has put forward a new possibility that androgen may act on prostate cancer cells through a novel GPCR dependent and AR

independent pathway. These results may provide a new explanation for progression of prostate cancer.

275 To sum up, this study explored the effect of androgens on  $[Ca^{2+}]_i$  in hormone-sensitive human prostate cancer cells, and examined the underlying mechanisms. The results suggest that androgens induced an L-type calcium channel dependent intracellular calcium increase in LNCaP prostate cancer cells. The rapid responses triggered by DHT did not appear to be mediated through classic intracellular androgen receptors or c-Src kinase-AR complex or SHBG, but through a GPCR. The precisely signal transduction pathways and effects of nongenomic  
280 action of androgen on prostate cancer cell proliferation, apoptosis or migration remain to be further investigated.

## Figure Legends

Fig1. Effects of DHT on  $[Ca^{2+}]_i$  in fura-2-loaded LNCaP cells. Concentrations of DHT were 1-1000 nM.

285 (A) DHT-induced  $[Ca^{2+}]_i$  increase in  $Ca^{2+}$  medium. Concentrations of DHT were 1 nM (●), 10 nM (○), 100 nM (□) and 1  $\mu$ M (▲), respectively. The black bar indicates the time of addition of DHT.

(B) Concentration-response plots of DHT-induced  $[Ca^{2+}]_i$  increase. Control is the basal  $[Ca^{2+}]_i$ . The y axis is the net maximum  $[Ca^{2+}]_i$  induced by DHT at different concentrations. Data are mean  $\pm$ SEM. \* $P$ <0.05, \*\* $P$ <0.001. Data are representative of at least six individual experiments.

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(C) Series of fluorescence ratio images, in pseudocolor, from a LNCaP cell preloaded with Fura-2/AM dye. The sequence shows a fast and transient fluorescence increase after testosterone addition. Scale bar=200  $\mu$ m.

295 Fig2. Source of intracellular calcium signal induced by androgens.

(A) Effect of extracellular  $\text{Ca}^{2+}$  removal and inhibitors of L-type  $\text{Ca}^{2+}$  channel on DHT-induced  $[\text{Ca}^{2+}]_i$  increase. Preincubation of LNCaP cells in calcium-free medium( $\square$ ) for 30 sec abolished the  $[\text{Ca}^{2+}]_i$  increase after stimulation with  $1\mu\text{M}$  DHT. Pretreatment with L-type calcium channel inhibitor,  $50\mu\text{M}$  Verapamil ( $\square$ ),  $100\mu\text{M}$  Diltiazem ( $\square$ ) or 300  $1\text{mM}$  Nifedipine ( $\bullet$ ) for 5min also blocked  $[\text{Ca}^{2+}]_i$  increase. The black bar indicates the time of addition of DHT.

(B) Incubation of cells with  $1000\text{nM}$  Neomycin sulfate ( $\square$ ; PLC inhibitors) for 5min or  $50\text{mM}$  Procaine ( $\square$ ; ryanodine receptor blocker) for 3min did not affect the calcium signal. The inhibitors remained present in the superfusion medium during the stimulation phase. The 305 black bar indicates the time of addition of DHT.

Fig 3. The mechanism of androgens-induced  $\text{Ca}^{2+}$  release.

- (A) The effect of CPA and Cycloheximide on the response of LNCaP cells to 1  $\mu\text{M}$  DHT. Cells were incubated for 3 h with 10  $\mu\text{M}$  Cycloheximide, a protein synthesis inhibitor, and then stimulated with DHT ( $\square$ ; 1  $\mu\text{M}$ ). Cycloheximide did not affect the  $\text{Ca}^{2+}$  increases induced by the hormone. Cells were incubated for 20 min with 1  $\mu\text{M}$  CPA, an antagonist of intracellular androgen receptor, and then stimulated with DHT ( $\square$ ). The use of CPA did not modify the  $\text{Ca}^{2+}$  increases produced by the hormone. The black bar indicates the time of addition of DHT.
- 310
- (B) Testosterone induced rise in  $[\text{Ca}^{2+}]_i$  of LNCaP cells. 10 nM Testosterone( $\square$ ) elicited an immediate  $[\text{Ca}^{2+}]_i$  increase in LNCaP cells. 10 nM Testosterone -BSA also increased  $[\text{Ca}^{2+}]_i$  ( $\blacktriangle$ ), while BSA( $\square$ ) was ineffective.
- 315

(C) Effects of genistein, GDPβS, and PTX on testosterone-induced intracellular Ca<sup>2+</sup> increases.

Cells were incubated with 100ng/ml PTX for 24 h and then stimulated with DHT (●; 1 μM). PTX blocked the Ca<sup>2+</sup> increases induced by the hormone. Cells were permeabilized  
 320 with saponin and stimulated with DHT (□). It is noted that in these conditions the cell did not lose the capacity to respond to the hormone. Nevertheless, permeabilization in the presence of GDPβS (500 nM) blocked the DHT induced Ca<sup>2+</sup> increases (□). Cells were incubated for 20 min with 100μM genistein, a tyrosine kinase inhibitor, and then stimulated with DHT (□). The use of genistein did not modify the Ca<sup>2+</sup> increases produced  
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# Figures

## Fig 1

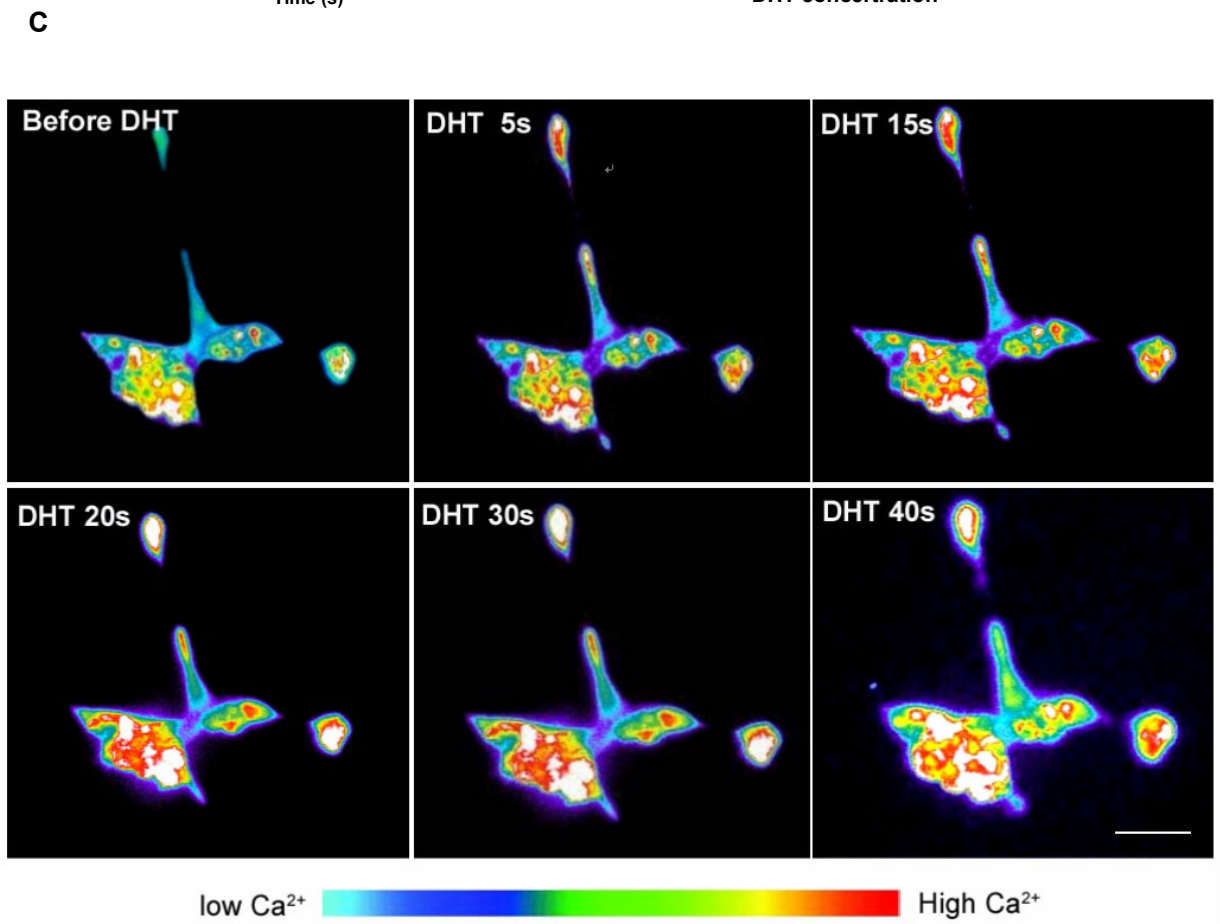
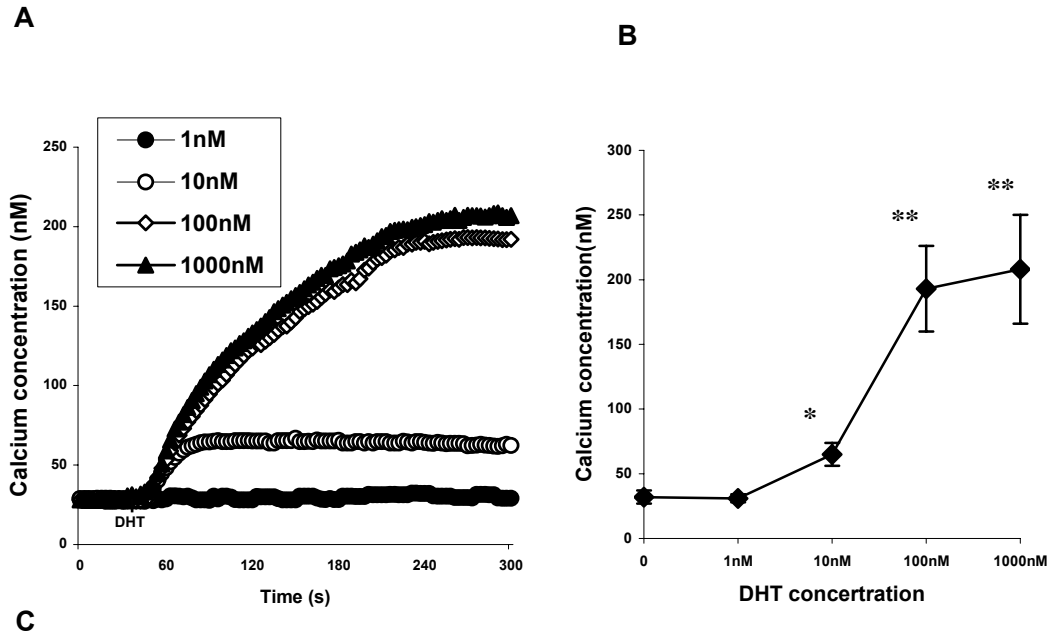


Fig2

A

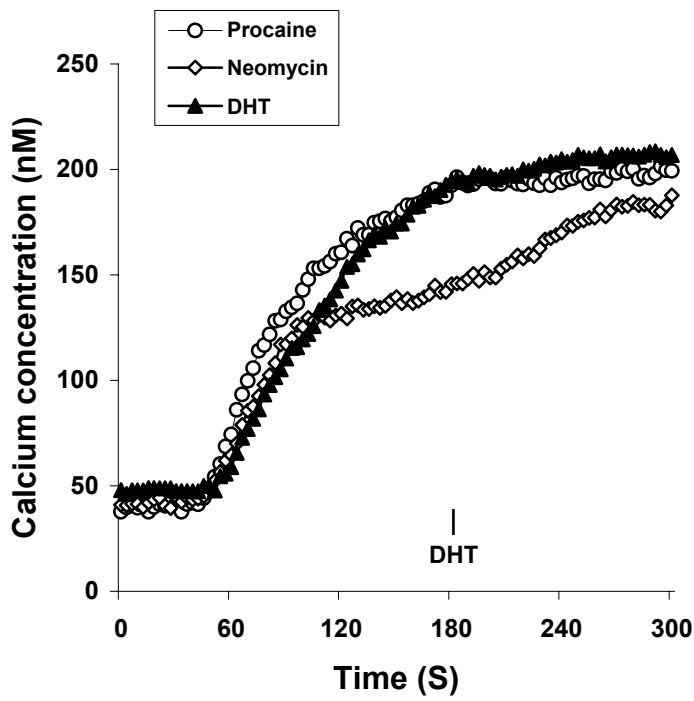
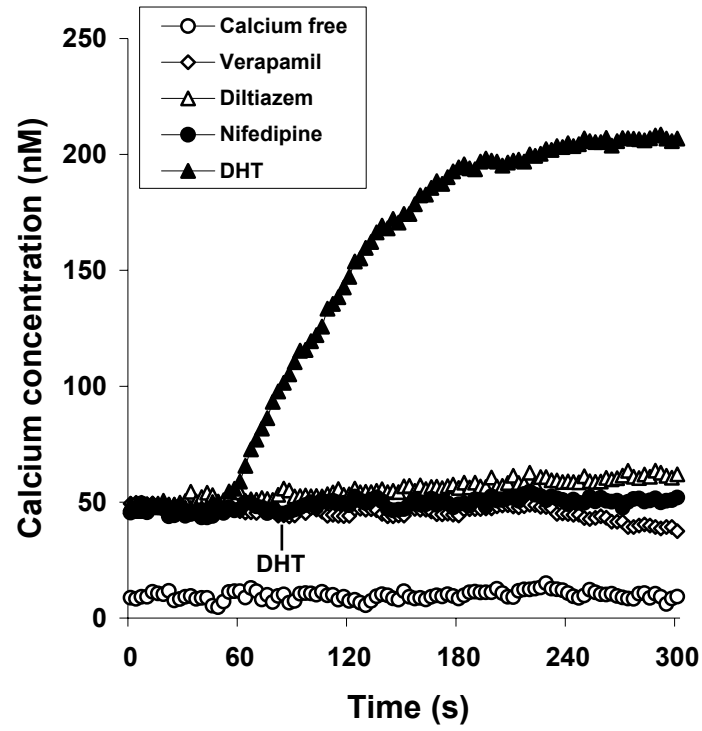


Fig 3

