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EFFECTS OF ANDROGEN ON INTRACELLULAR CALCIUM OF LNCaP CELLS

Jaime Steinsapir,* Robin Socci and Peter Reinach

Department of Physiology and Endocrinology
Medical College of Georgia
Augusta, GA 30912

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Mibolerone (dimethylnortestosterone) or 5α-dihydrotestosterone (DHT) increase intracellular calcium (Ca_i^{2+}) of human prostate cancer cells (LNCaP) as early as 2 min after treatment. These effects were concentration-dependent (10^{-6}-10^{-12}M) and they were blocked by preincubation with hydroxyflutamide (10^{-6}M). Verapamil (10^{-6}M) also suppressed the mibolerone (10^{-6}M)-induced increase in Ca_i^{2+}, in cells which were previously exposed to 1mM $CaCl_2$ introduced in a Ca^{2+}-free media. The results indicate that androgens elicit changes in Ca_i^{2+} in LNCaP cells as a result of Ca^{2+} influx through L-type channels in the plasma membrane. Since androgens are involved in the regulation of prostate cell division and growth, these findings suggest that calcium is involved in metabolic and mitogenic responses to steroid hormone in target cells.

Steroid hormones control cell division and growth in target cells [1,2]. In view of evidence suggesting a critical role for calcium in the initiation and/or regulation of cell growth and metabolism [3,4], consideration of its involvement as a second messenger is essential to determine how steroids control these processes. There is accumulating evidence from several groups which indicate that steroid hormones may trigger plasma membrane-mediated events in target cells [1,2], because estrogens promote a rapid increase (2.5 min) of the rate of calcium exchange in endometrial cells isolated from uteri of ovariectomized rats [1]. However, little is known about how androgens may control Ca_i^{2+} levels in normal and neoplastic target cells. LNCaP cells are a widely used model to study androgen action [5] in responsive cancer cells. The main aims of this work were: a) to study the effects of 5α-DHT or mibolerone on Ca_i^{2+} of LNCaP cells; b) to determine the effects of hydroxyflutamide on Ca_i^{2+}, in the presence or absence of androgen and c) to assess the effects of verapamil, a L-type calcium channel blocker [6], on the androgen-induced changes of Ca_i^{2+} in LNCaP cells, in vitro.

*Author to whom correspondence should be addressed.

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MATERIALS AND METHODS

Materials: Mibolerone (dimethylnortestosterone) was purchased from Amersham, Searle. Radioinert 5α-dihydrotestosterone was obtained from Sigma (St. Louis, MO). Hydroxyflutamide (2-hydroxy-2-methyl-N-[4-nitro-3-(trifluoro-methyl)phenyl]propanamide, SCH-16423) was from Schering Corp., Bloomfield, NJ, U.S.A. Fura-2 was purchased from Molecular Probes (Eugene, OR). Media, sera and antibiotics used to culture the LNCaP cells were obtained from Grand Island Biological Co. (Grand Island, NY). Ca^{++}-free RPMI 1640 was from JR Scientific, Woodland, CA. All other chemicals were of analytical grade.

Cell culture: Cells were grown in RPMI 1640 medium (GIBCO) with gentamicin (50 mg/ml) and 5% heat-inactivated fetal bovine serum (FBS), at 37°C in a humidified atmosphere of 5% CO_2 in air. The cultures were fed at 3 to 4-day intervals with fresh medium in 75-sq. cm. plastic flasks (Falcon Plastics, Oxnard, CA). LNCaP cells between 10 and 17 passages in vitro were used to conduct these studies. The results of radioimmunoassay analysis of FBS indicated the following steroid levels: testosterone, 0.28 nM; progesterone, 0.127nM; 17β-estradiol, 0.125 nM. FBS contains 3.75 mM Ca^{2+} (GIBCO). RPMI 1640 contains 0.42 mM $Ca(NO_3)_2x4H_2O$.

(Ca_i^{2+}) measurements: Cells were harvested by incubation at 37°C for 10 min in trypsin (0.05%) and EDTA (0.02%) in phosphate buffer (0.05 M KH_2PO_4, 0.15M NaCl, pH 7.2 at 22°C). Trypsin reactions were stopped by 1:3 dilution with FBS-containing RPMI 1640. The cells were then washed three times in this medium at 600 xg for 6 min. and washed twice in serum-free RPMI 1640. Cells were counted and their viability was assessed with Erythrosin B. LNCaP cells were loaded with 3μM Fura -2/AM [7,8] at 34°C for 45 min in RPMI 1640. Following three washes at 600 xg for 6 min with the same buffer used for cell loading with Fura-2, the cell pellets were resuspended in the appropriate buffer (i.e. Ca^{2+} containing or Ca^{2+}-free/buffer) at a cell density of $1x10^6$ cells/ml. Washed cells were added to 1 cm cuvettes ($2x10^6$ cells/cuvette/2 ml) in a Delta Scan-1 spectrofluorometer. (Photon Technology International Inc., South Brunswick, NJ). After a control period, any changes upon addition of vehicle (0.25% (v/v) ethanol) were determined before the addition of agonist or antagonist. After each experiment, the maximum fluorescence was checked with the introduction of 10^{-5}M ionomycin. Any fura-2 leakage, the maximum and the minimum fluorescence (Fmax and Fmin) were determined by the addition of 0.15mM $MnCl_2$, 10^{-5}M ionomycin, 1.25mM DTPA (pentetic acid), 5.0 mM EGTA or 50mM $CaCl_2$ to cuvettes. The fluorescence emission ratios at 510 nm were determined following alternating excitation at 340 and 380nm. Corrections were performed for the background fluorescence of unloaded cells, in each experiment.

Calcium concentrations were calculated based on calibration curves that were constructed from the fluorescence ratios (340 nm/380 nm) of solutions which contained different Ca^{2+}/EGTA ratios and the appropriate buffer for pH 7.4 [9,10]. The apparent association constant, K_d for Fura-2 interactions with Ca^{2+} was 222nM, which is in agreement with the range of values (135 to 225nM) reported by Grynkiewicz et al [9,11]. Results were expressed as percentages of increase in intracellular calcium concentration (nM), in each experiment: [(Ca_i^{2+}after stimuli - Ca_i^{2+} control)/Ca^{2+} control)]. These percentage values were corrected for the percentage increase in Ca_i^{2+} due to the vehicle alone.

RESULTS

Testosterone is metabolized to its active metabolite DHT in prostate cells [12] which was used to examine the effects of androgen on Ca_i^{2+} in LNCaP cells. Figure 1 shows that DHT increased from 14nM to 88nM in 1-2 min. Furthermore, this increase was concentration-dependent because neither 10^{-9} nor 10^{-12}M DHT increased Ca_i^{2+} (Figure 2). It has been reported that DHT can be partially metabolized by LNCaP cells, in vitro [13]. We used an stable, synthetic androgen, mibolerone [14,15] in subsequent studies. Figure 3 shows that mibolerone also induced a concentration-dependent increase of Ca_i^{2+}. We observed that this response reached a "plateau" that remained stable for as long as 5 min, in the presence of 10^{-6}M mibolerone. Furthermore, when the cell medium was supplemented with 1 mM $CaCl_2$, mibolerone increased Ca_i^{2+} at higher levels than those observed in a medium which was not supplemented with calcium (Figure 3).

Since calcium is important for prostate cell growth [16,17] and androgens as well as antiandrogens increase LNCaP cell growth [18,19], it was of interest to analyze the effects of hydroxyflutamide on Ca_i^{2+} in the presence or absence of mibolerone, in this cell system. Figure 4 indicates that hydroxyflutamide alone increased intracellular calcium in a Ca^{2+} concentration dependent manner. Although 10^{-6}M mibolerone increased Ca_i^{2+}, the addition of 10^{-3}M hydroxyflutamide immediately before 10^{-6}M mibolerone effectively blocked the androgen-induced increase in Ca_i^{2+}.

Two different types of experiments were performed to delineate the source of an increase in Ca_i^{2+}. In the first type, the cells were bathed in a Ca^{2+}-free medium, either in the presence or absence of 0.2mM EGTA. In the second type, the cells were bathed instead in the

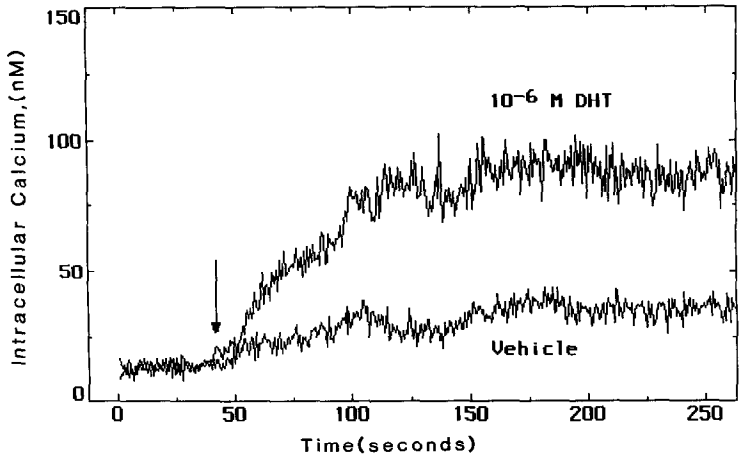

Figure 1. Effect of 5α-dihydrotestosterone (DHT) on the intracellular calcium concentration (nM) of LNCaP cells. Cells (1 x 10^6 cells/ml) were exposed to 10^{-6}M DHT or vehicle (ethanol, final concentration = 0.25% v/v). The fura-2 fluorescence ratio (340/380) was measured and intracellular calcium quantitated as indicated in "Materials and Methods".

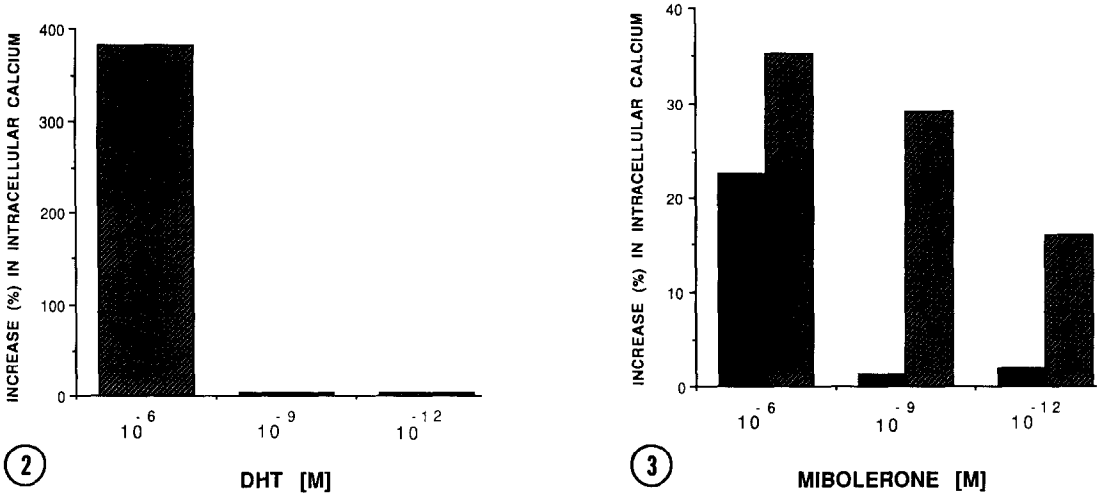

Figure 2. Effects of different concentrations of DHT on Ca_i^{2+} of LNCaP cells in the presence of RPMI 1640. Ca_i^{2+} was measured as indicated in "Materials and Methods". Experimental values were corrected for vehicle values shown in Figure 1. The same effect of 10^{-6}M mibolerone was observed in three different experiments.

Figure 3. Effects of different mibolerone concentrations on Ca_i^{2+} of LNCaP cells, in the presence of a phosphate-saline buffer (50 mM KH_2PO_4, 0.15 M NaCl, pH 7.2 at 22°C) supplemented or not with 1 mM Ca Cl_2 (■: - Ca Cl_2, ▨: + Ca Cl_2). Ca_i^{2+} was measured as indicated in "Material and Methods". Each percentage value is shown after subtraction for the vehicle effect (25.5% over basal Ca_i^{2+}).

Ca^{2+}-containing medium which also contained 10^{-6}M Verapamil. The results shown in Figure 5 indicate that mibolerone did not increase Ca_i^{2+} in the absence of calcium in the external bath. Mibolerone was also ineffective in the Ca^{2+}-free medium supplemented with 0.2mM EGTA. The requirement for mibolerone to elicit an increase in Ca_i^{2+} is clearly dependent on external Ca_i^{2+} because after subsequent addition of 1mM $CaCl_2$ the mibolerone-induced rise in $Ca_i^{2+}$was restored (Figure 5). That the Ca^{2+} influx elicited by mibolerone in LNCaP cells occurs through a voltage dependent L-type Ca^{2+} channel pathway in the plasma membrane is shown by the effect of preincubation of the cells with 10^{-6}M verapamil (Figure 5). As in the Ca^{2+}-free system, mibolerone failed to elicit a significant increase in Ca_i^{2+}. Therefore, the increase in Ca_i^{2+} following exposure to mibolerone stems from Ca^{2+} influx from the external bath through a voltage dependent Ca^{2+} channel (L-type).

DISCUSSION

Little is known about the mechanisms by which steroid hormones induce the so-called "early" responses in responsive tissues and cells although numerous "early" responses to steroid hormones have been previously described in target tissues [20,21]. For example, estrogens

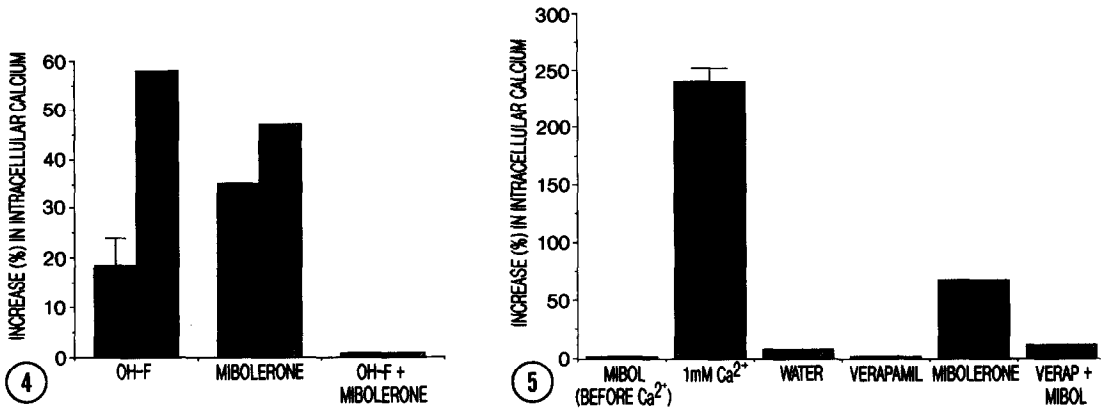

Figure 4. Effects of hydroxyflutamide (10^{-3}M) and/or mibolerone (10^{-6}M) on Ca_i^{2+} of LNCaP cells, in the presence of a phosphate-saline buffer (indicated in legend of figure 3) supplemented with 1 mM (■) or 2 mM (▨) Ca Cl_2. Cytosolic calcium concentrations were measured as indicated in "Materials and Methods". Each percentage value is shown after subtraction for the vehicle effect (26.1% over basal Ca_i^{2+}). The bar shows the range of values of two different determinations obtained in two different cuvettes from the same batch of cells. The same effects of hydroxyflutamide were observed in two different experiments.

Figure 5. Effects of verapamil (10^{-6}M) and mibolerone (10^{-6}M) on Ca_i^{2+} of LNCaP cells. Fura-2 loaded cells were suspended in Ca^{++}-free RPMI 1640 and exposed to mibolerone or vehicle (ethanol, 0.25% v/v). After addition of 1 mM $CaCl_2$, cells were treated with verapamil, water (vehicle of verapamil), mibolerone (10^{-6}M) again or verapamil (10^{-6}M) immediately before 10^{-6}M mibolerone. Ca_i^{2+} was measured in each condition as indicated in previous experiments. Values were corrected for the vehicle (ethanol) effect (25.0% over basal Ca_i^{2+}). The bar shows the range of values of two different determinations obtained in two different cuvettes from the same batch of cells. The same effect of verapamil was observed in two different experiments.

induce early changes in the content of water and divalent electrolytes in the uterus [1] but there is no information available on the prostatic calcium content shortly after androgen stimulation. Moreover, although we know that increments in cellular calcium accumulation precede and/or trigger cell division and growth [3,4], which is one end-point of androgen action in responsive cells, we do not know if calcium serves as a second messenger for androgen action in prostatic cancer cells. The findings reported here that androgen acutely increases cytosolic calcium of LNCaP cells are of significance to characterize the processes involved in metabolic and mitogenic responses to the hormone.

The LNCaP cells were selected as a model to study early events in androgen action since we know that the growth of these cells is androgen-dependent and that they do not lose androgen responsiveness with time in culture [5]. These properties, as well as the presence of androgen receptor (AR) in these cells [22], make them the most promising in vitro model of human prostate cancer currently available. Prostate cell death and proliferation have been both

associated with alterations in intracellular calcium levels [17]. Treatment of castrated rats with calcium channel blocking drugs induced a delay in rat prostate regression in vivo, as well as induction of c-fos mRNA levels [17]. The finding that androgen effects on intracellular calcium levels of LNCaP cells are fast (2 min.) makes it unlikely that AR is involved in this acute androgen response. Although hydroxyflutamide was able to block mibolerone-induced rises in cytosolic calcium, it is likely that both androgen and antiandrogen interact with plasma membrane components of the LNCaP cell to mediate changes in intracellular calcium concentration. The LNCaP cells may have voltage-dependent calcium channels which are controlled by androgen and antiandrogen, since it is known that steroid hormones may change the polarization state of target cell membranes [20]. Several reports have described the existence of plasma membrane binding sites for steroid hormones [23,24]. However, these results have not been reproducible in the hand of others [25]. These calcium channels provide an alternative mechanism for androgen transport distinct from the classical passive diffusion mechanism of steroid entry into target cells [26].

Receptor sites for androgens have been described to be associated with microsomal membrane components other than plasma membranes [25]. The results reported here indicate that androgens increase the cytosolic calcium of LNCaP cells by mechanisms dependent upon the presence of extracellular calcium. However, androgens could also induce redistribution of Ca^{++} at intracellular sites. Microsomal AR could be important to mediate androgen-induced increases in intracellular calcium dependent upon intracellular calcium sources. It has been also reported that 5αDHT, RU23908, cyproterone acetate and hydroxyflutamide are able to increase phosphoinositide (PI) turnover 12-24 hours after stimulation of LNCaP cells [27]. Phospholipase C activity also increases after 5αDHT treatment of LNCaP cells [27]. Although the previously described responses in PI turnover are usually secondary to a rise in cytosolic calcium from an intracellular origin, it is also possible that Ca^{2+} influx from extracellular sources may increase PI turnover in neoplastic prostate cancer cells. The acute (2 min) androgen - induced rises in cytosolic calcium of LNCaP cells reported here may provide an explanation for the late (12 hours) changes observed in phosphoinositide metabolism of LNCaP cells previously mentioned [27]. Furthermore, the results shown here with hydroxyflutamide indicate that this "antiandrogen" has both agonistic and antagonistic properties in the control of intracellular calcium in this neoplastic cell system, in agreement with previously reported agonistic effects of hydroxyflutamide on phosphatidylinositol metabolism [27] and LNCaP cell growth [18,19]. Taken together, these findings may indicate a role for calcium in the mechanisms of androgen action and its regulation of LNCaP cell growth and metabolism.

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