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Cell surface effects of androgens between 1 pM and 100 nM on rat sertoli cells and two human prostatic cell lines, LNCaP and PC3: evidence for two membrance receptors

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Rapid Androgen Actions on Calcium Signaling in Rat Sertoli Cells and Two Human Prostatic Cell Lines: Similar Biphasic Responses Between 1 Picomolar and 100 Nanomolar Concentrations[1]

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ABSTRACT

Androgen-induced calcium fluxes and gap junctional intercellular communication (GJIC) were studied in three different cell types. A transient (2–3 min duration) increase in intracellular calcium levels was observed within 20–30 sec of androgen addition, which was followed by a plateau phase with steroid concentrations higher than 1 nM. The kinetics of the calcium responses were similar in immature rat Sertoli cells, which contain normal nuclear receptors; the human prostatic tumor cell line, LNCaP, which contains a mutated nuclear receptor; and the human prostatic cell line, PC3, which does not contain a nuclear receptor. The human A431 tumor cell line did not respond to androgens. Concentrations of testosterone and the synthetic androgen, R1881, between 1–1000 pM induced transient calcium increases with ED_{50} values near 1 pM and 1 nM, whereas dihydrotestosterone (DHT) was not active at these concentrations. At concentrations higher than 1 nM, testosterone, R1881, and DHT were equipotent in stimulating an increase in calcium that lasted for more than 10 min, with ED_{50} values between 5 and 20 nM. Testosterone covalently bound to albumin was also active, whereas 11 related androstane compounds as well as progesterone and estradiol-17β were inactive at 1000 nM. The calcium response induced by the three androgens (10 nM) was abolished in all cell types by hydroxyflutamide (1000 nM) and finasteride (1000 nM), but not by cyproterone acetate (1000 nM). The calcium response was also abolished in the absence of extracellular calcium and strongly inhibited by the presence of verapamil. Exposure of the responsive cells to brief (150-sec) pulses of androgens generated calcium responses that were similar to those after continuous exposure. After exposure of Sertoli cells for only 30 sec to 100 nM testosterone, the calcium response lasted for at least 50 min. Although nuclear binding of androgens could be demonstrated, there was no evidence for tight binding to the plasma membrane under similar conditions. When protein synthesis was inhibited, an enhancement of GJIC between rat Sertoli cells, but not between LNCaP cells or PC3 cells, was observed within 15 min of the addition of 10 nM testosterone. Because nuclear androgens are not present in PC3 cells and many functional properties of the responsive system are different from the nuclear receptor in all three cell types, we postulate the existence of an alternative cell surface receptor system with biphasic response characteristics (high and low affinity). The calcium signals are probably coupled to the regulation of gap junctional efficiency between Sertoli cells. The low-affinity receptors may convey complementary androgen signals at elevated local levels such as in the testis, when nuclear receptors are (over)saturated.

calcium, hormone action, prostate, Sertoli cells

INTRODUCTION

Spermatogenesis occurs when testicular levels of androgens are much higher than peripheral androgen levels [1, 2]. Although the discussions about the minimal amount of testosterone that is required for maintenance of spermatogenesis are still ongoing, there is no doubt that concentrations higher than the normal peripheral levels of testosterone are required [3, 4]. Because Sertoli cells are central to the regulation of spermatogenesis, it is possible that specific Sertoli cell functions can be sufficiently stimulated only by these high concentrations of androgens. This hypothesis is supported by the observation that androgen binding protein (ABP) secretion in vitro by a combination of rat Sertoli cells and peritubular myoid cells can be stimulated only by free testosterone doses of between 4 and 20 nM [5], whereas androgen-responsive cells derived from peripheral organs, such as LNCaP cells and cell lines that have been transfected with the androgen receptor, show a full response to androgens between 0.1 and 1 nM [6].

The major mechanism of action of androgens is via high-affinity receptors in the nucleus through activation of DNA transcription. The saturation level of nuclear high-affinity androgen receptors is close to 1 nM, which is in accordance with many observed biological responses. It is difficult to envisage how these high-affinity receptors can sense and respond further to androgen levels above their saturation point. An independent low-affinity transducing system, acting in conjunction with the high-affinity receptors, may explain the odd response characteristics of testicular cells [7]. Supporting such an alternative receptor system is the observation that in vitro spermatogonial multiplication in eels can be stimulated only with 11 keto-testosterone, for which no nuclear receptor has been identified, whereas testosterone and dihydrotestosterone (DHT), for which nuclear receptors have been identified, were not active [8].

In recent years, a variety of membrane actions of steroids have been reported for otherwise nuclear oriented ligands, such as progesterone [9, 10], estrogens [11, 12], corticosterone [13], aldosterone [14, 15], and vitamin D [16, 17]. This is also discussed in a general review by Wehling [18] and, more specifically, for reproductive organs by Revelli et al. [19]. Many of these steroids can induce transient elevations of intracellular calcium within 1 min, and many authors have explained these phenomena by postulating the existence of receptors for steroids in the plasma membrane that differ from the nuclear receptors.

Androgens can also induce rapid calcium fluxes in a va-

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riety of cell types, including human prostatic cancer cells (LNCaP; [20]), rat heart myocytes [21], male (but not female) rat osteoblasts [22], and mouse T cells, in which the presence of a functional classical androgen receptor could not be demonstrated [23, 24]. Recently, it was shown that human granulosa cells show a calcium response to androstenedione but not to testosterone [25]. Furthermore, Gorczynska and Handelsman [26] have shown rapid calcium fluxes following addition of high concentrations of androgens to freshly isolated immature rat Sertoli cells. However, the doses of testosterone required for a response (between 300 and 3000 nM) were far in excess of the already high local testicular levels (estimated free testosterone 20–60 nM [2]). This poor response of the freshly isolated cells may have been influenced by membrane damage during the enzymatic isolation procedure, as has been shown for glucocorticoid receptors in the plasma membrane of lymphoma cells [27].

In an effort to understand the mechanisms of action of androgens, we have investigated various characteristics of androgen-induced calcium signaling and as an immediate functional expression of this regulatory pathway, gap junctional intercellular communication (GJIC). Because there are observations that estrogen receptors (perhaps modified) can activate signaling pathways at the plasma membrane [28, 29] and, because suggestions have been made that this could hold also for androgen receptors [30], we have not only investigated rat Sertoli cells, but also the human prostate cancer cell line, LNCaP, which possesses a mutated androgen receptor [6], and the androgen-insensitive human prostate cancer line, PC3, in which mRNA for the androgen receptor could not be detected [31].

MATERIALS AND METHODS

Primary Culture of Seminiferous Tubules

Testes from 21- to 25-day-old Wistar rats (Biological Services Unit, University of Manchester, UK) were collected in 10 ml Dulbeccos PBS containing 1 mM Ca^{2+} and 1 mM Mg^{2+}. The tunica albuginea was removed and the tissue was incubated in 1 mg/ml collagenase (type 1A, activity >125 collagen digestion units per mg solid; Sigma Chemical Company, St. Louis, MO) in PBS containing 1 mM Ca^{2+} and 1 mM Mg^{2+} at 37°C for 30 min. Incubation was terminated by addition of 10 ml culture medium containing 1% fetal calf serum (FCS; Gibco, Paisley, Strathclyde, UK) and pieces of seminiferous tubules were collected after sedimentation at gravity. One drop of a concentrated suspension of tubular fragments was added to a glass coverslip in a culture dish with RPMI 1640 medium without phenol red (Sigma) containing 1% FCS. Tubules were cultured for 1–4 days at 37°C in a humidified atmosphere of 5% CO_2. During this period the Sertoli cells migrated away from the explants. Close to the remnants of the tubular fragments, clusters of germ cells remained attached to the flat monolayer of Sertoli cells and, at the periphery, only a monolayer of Sertoli cells existed [32].

Culture of Cell Lines

LNCaP cells [33] and PC3 cells [34] were grown in normal RPMI 1640 medium without phenol red (Sigma) supplemented with 7.5% FCS. The human epidermal carcinoma cell line, A431 [35], was grown in normal DMEM without phenol red (Sigma) supplemented with 10% FCS. All cells were incubated for 2–3 days at 37°C in a humidified atmosphere of 5% CO_2 before they were used for measurements.

Chemicals

Testosterone (4-androsten-17β-ol-3-one), androstenedione (4-androstene-3,17-dione), epitestosterone (4-androsten-17α-ol-3-one), 5β-dihydrotestosterone (5β-androstane-17β-ol-3-one), 5α-dihydrotestosterone (5α-androstane-17β-ol-3-one), 5α-androst-16-en-3α-ol, estradiol-17β (1,3,5(10)-estratriene-3,17β-diol), progesterone (4-pregnene-3,20-dione), 11β-hydroxyandrostenedione (4-androsten-11β-ol-3,20-dione), dehydroepiandrosterone (5-androsten-3β-ol-17-one), 5β-androstan-3β-ol-17–one, 5α-androstane-3,17-dione, 5α-androst-3-one, 5α-androst-16-en-3-one, and 5α-androst-16-en-3β-ol were all obtained from Steraloids (Newport, RI). R1881 (17β-hydroxy-17α-methyl-estra-4,9,11-trien-3-one) was a gift from Roussel UCLAF (Romainville, France). Cyproterone acetate and hydroxyflutamide (HF) were gifts from Schering (Berlin, Germany). Human recombinant hFSH (Org 32489) was a gift from Organon (Oss, The Netherlands). Finasteride, testosterone 3-(O-carboxy methyl)oxime:BSA (29 mol steroid/mol BSA), testosterone 3-(O-carboxy methyl) oxime BSA-fluorescein isothiocyanate conjugate (FITC), bovine albumin-fluorescein isothiocyanate, epidermal growth factor (EGF), cycloheximide, lucifer yellow CH, and the ionophore A23187 were obtained from Sigma. All compounds were dissolved in ethanol. Fluo 3 and Fura Red acetoxymethyl (AM) ester (Molecular Probes, Leiden, The Netherlands) were dissolved in DMSO. All dilutions were made in buffer solutions so that the final concentration of ethanol or DMSO was less than 0.1%.

Loading With Fluo 3 and Fura Red AM Esters

Cultures were washed twice with a buffer containing 130 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 25 mM Hepes (pH 7.4). Cells were loaded with the calcium-sensitive dyes by incubation with 3 μM Fluo 3 and 3 μM Fura Red AM esters for 1 h in the buffer at 37°C. Subsequently, the cultures were washed three times with buffer and the glass coverslip carrying the cells was mounted in a temperature-controlled microperfusion chamber constructed of aluminum with a volume of 1 ml. It was possible to rapidly change conditions with a perfusion rate of 2 ml/min. At a constant perfusion, a steady state concentration of ligands could be maintained. This is especially important when ligands can be metabolized by the cells. In some experiments, cells were preincubated for 20 min with inhibitors prior to calcium measurements.

Ratiometric Measurement of Calcium

The fluorescence intensity from a 100-μm^2 area, corresponding to approximately 10 fluorescently loaded cells, was measured using a high-sensitivity low-irradiance microfluorimeter [36]. Fluo 3 and Fura Red [37–39] were excited at 488 nm using light from the Daresbury Laboratory synchrotron radiation source, which provided long-term stability and low irradiance. Fluorescence emissions were simultaneously recorded at 525 nm and 660 nm. Fluorescence intensities were integrated every 2 sec for periods of 15 min with hormones being added 2 min after the start of the measurement. The data were analyzed using in-house data analysis software (http://www.dl.ac.uk/SRS/NCD/manual.otoko.html) to generate the Fluo 3/Fura Red fluorescence ratios. Because ratios could be calculated imme-

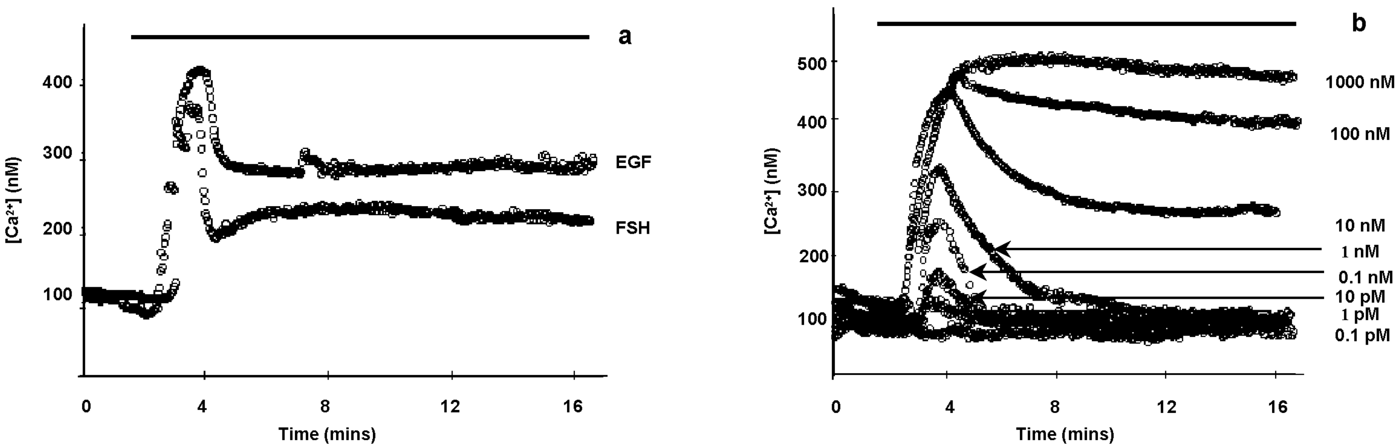

FIG. 1. Kinetics of calcium response after hormonal stimulation of Sertoli cells. Intracellular calcium levels in rat Sertoli cells before and during perfusion with **a**) EGF (100 nM) or FSH (100 nM) and **b**) testosterone (0.1 pM–1000 nM). The black bar indicates the presence of hormone in the perfusion medium. Ca^{2+} was measured after loading cells with calcium-sensitive fluorescent dyes, Fluo 3 and Fura Red, as indicated in *Materials and Methods.* Data are representative of at least six individual experiments. Similar curves for androgens were obtained when human LNCaP cells or PC3 cells were used.

diately, a group of cells that gave a constant ratio could be easily positioned under the objective lens.

Correlation of the Fluo 3/Fura Red fluorescence ratios to absolute calcium concentrations was performed using a 2-point in situ calibration method similar to that described by Foskett et al. [40]. The minimum fluorescence ratio (R_{min}) for Fluo 3 and Fura Red in the absence of calcium was determined from the fluorescence of Fluo 3/Fura Red-loaded cells exposed to 5 μM ionophore A23187 in a zero Ca^{2+} buffer containing 10 mM EGTA. Normal intracellular calcium levels were restored within 30 sec when cells exposed to zero calcium were perfused again with normal buffer containing 1 mM calcium in the absence of ionophore. This indicates that the effects of the ionophore under these conditions are reversible. The maximum fluorescence ratio (R_{max}) was determined from the fluorescence of cells exposed to 5 μM ionophore A23187 in a 10 mM Ca-EGTA buffer with 600 nM free-Ca^{2+}.

Binding of Androgens

Binding of tritium-labeled R1881 was measured in intact cells as described previously [41]. With this technique, specific, low-capacity binding to the nuclear receptor can be discriminated from nonspecific high-capacity binding to cellular proteins by comparing cellular binding in the absence and presence of an excess, unlabeled steroid.

Scrape Loading-Dye Transfer Technique

Cultures were washed in PBS and incubated in 0.05% lucifer yellow CH in PBS. Scrape lines were made with a needle in the cell monolayer and the cultures were left undisturbed for 3 min. The cultures were washed several times with PBS and examined with a Zeiss epifluorescence microscope. The fluorescent dye enters the primary cells on either side of the scrape line and is subsequently transferred through gap junctions into adjoining cells. The extent of dye transfer from the primary cells to the adjoining cells gives a measure of GJIC [42]. The percentage of fluorescently labeled cells in a defined area was used as a quantitative measure of GJIC in control and treated cells.

Data Analysis

Measurements of Ca^{2+} are presented as mean values ± SEM of *n* independent experiments, each performed in triplicate. Significance of differences was determined by an unpaired Student's *t*-test and the differences were considered significant if $P < 0.001$.

RESULTS

Measurement of Calcium and Effects of Protein Hormones

At the start of the experiment, the Fluo 3/Fura Red fluorescence ratio values varied between 0.3 and 1.3. This could indicate either cellular heterogeneity in calcium levels or variations in dye loading. Therefore, various cell areas were prescreened to select a group of approximately 10 cells that gave the standard starting ratio value of 0.5, which was the mean of the distribution.

In order to test the analytical system and the responsiveness of the cells, calcium responses were measured after exposure of the cells to protein hormones because it is known that Sertoli cells respond to FSH and EGF [43], whereas LNCaP [44] and A431 cells [35] respond only to EGF. Figure 1a shows the typical rapid response pattern of Sertoli cells to high concentrations of EGF and FSH: a transient calcium peak followed by a plateau phase higher than basal level, but lower than the peak level.

Kinetics and Dose-Response Properties of Androgens

Rapid increases in calcium levels were also found following addition of androgens. Typical responses for testosterone are shown in Figure 1b for rat Sertoli cells. Similar responses were seen for LNCaP cells and PC3 cells. A431 cells did not respond to androgens and served as controls. In all responding cells, different kinetics were observed, depending on the dose of androgen used. In general, low concentrations of androgens generated a transient peak lasting 2–3 min after a delay of approximately 20 sec. The lowest dose of testosterone and R1881 to show a response was 1 pM. Higher androgen concentrations caused an increase in the peak height. At concentrations higher than 10 nM testosterone, R1881, or DHT for rat Sertoli cells and 1

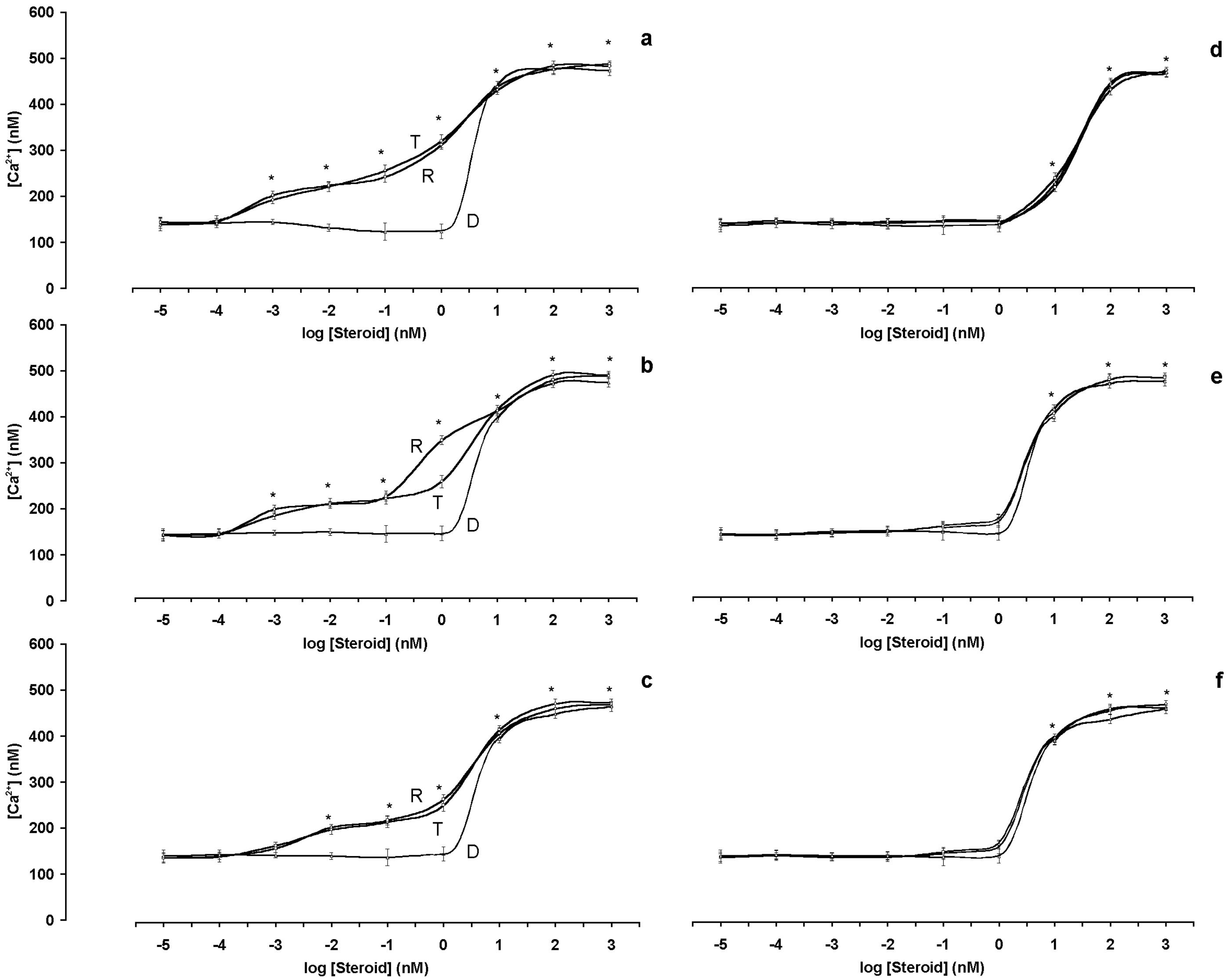

FIG. 2. Dose dependence of calcium responses. Dose-response curves for 0.1 pM–1000 nM testosterone (T), R1881 (R), and DHT (D) were constructed from intracellular calcium levels 2 min after hormone addition (**a**–**c**) and 10 min after hormone addition (**d**–**f**) for rat Sertoli cells (**a**,**d**), human LNCaP cells (**b**,**e**), and human PC3 cells (**c**,**f**). Mean values ± SEM of three individual experiments performed in triplicate are given. * $P < 0.005$ compared with controls.

nM testosterone, R1881, and DHT for LNCaP and PC3 cells, a persistent elevation of calcium after the transient peak was observed. The response pattern of cells stimulated with DHT was different from that of testosterone or R1881, because concentrations of DHT below 1 nM did not affect intracellular calcium. We constructed two dose-response curves from the kinetic data of each cell type, one describing the transient peak levels at approximately 2 min after hormone addition, and one describing the dose-dependent plateau levels after approximately 10 min (Fig. 2). The dose-response curves of the three cell types were very similar and a biphasic stimulation pattern could be recognized. Approximate ED_{50} values of the two phases of stimulation in the three cell types are given in Table 1.

TABLE 1. Approximate ED_{50} values[a] of testosterone, R1881, and dihydrotestosterone for half-maximal stimulation of high- and low-affinity receptive systems in rat Sertoli cells, LNCaP cells, and PC3 cells.

	Transient high-affinity[b]			Transient low-affinity[c]			Steady-state low-affinity[d]		
Ligand	T	DHT	R1881	T	DHT	R1881	T	DHT	R1881
Sertoli cells	0.5 pM	—	0.5 pM	2 nM	5 nM	2 nM	20 nM	20 nM	20 nM
LNCaP cells	0.5 pM	—	0.5 pM	5 nM	5 nM	500 pM	5 nM	5 nM	5 nM
PC3 cells	5 pM	—	5 pM	5 nM	5 nM	5 nM	5 nM	5 nM	5 nM

[a] The approximate ED_{50} values were estimated from the dose response curves in Figure 2 at the half-maximal stimulation of intracellular calcium which is the level between two steady-state levels. DHT, Dihydrotestosterone; T, testosterone.

[b] Levels are the basal level and the intermediate calcium plateau, when androgen concentrations are between 10 and 100 pM.

[c] Levels are the intermediate calcium plateau at 10–100 pM androgens and the maximal calcium concentration at saturating androgen concentrations.

[d] Levels are (right curves in Fig. 2) the basal and maximal level.

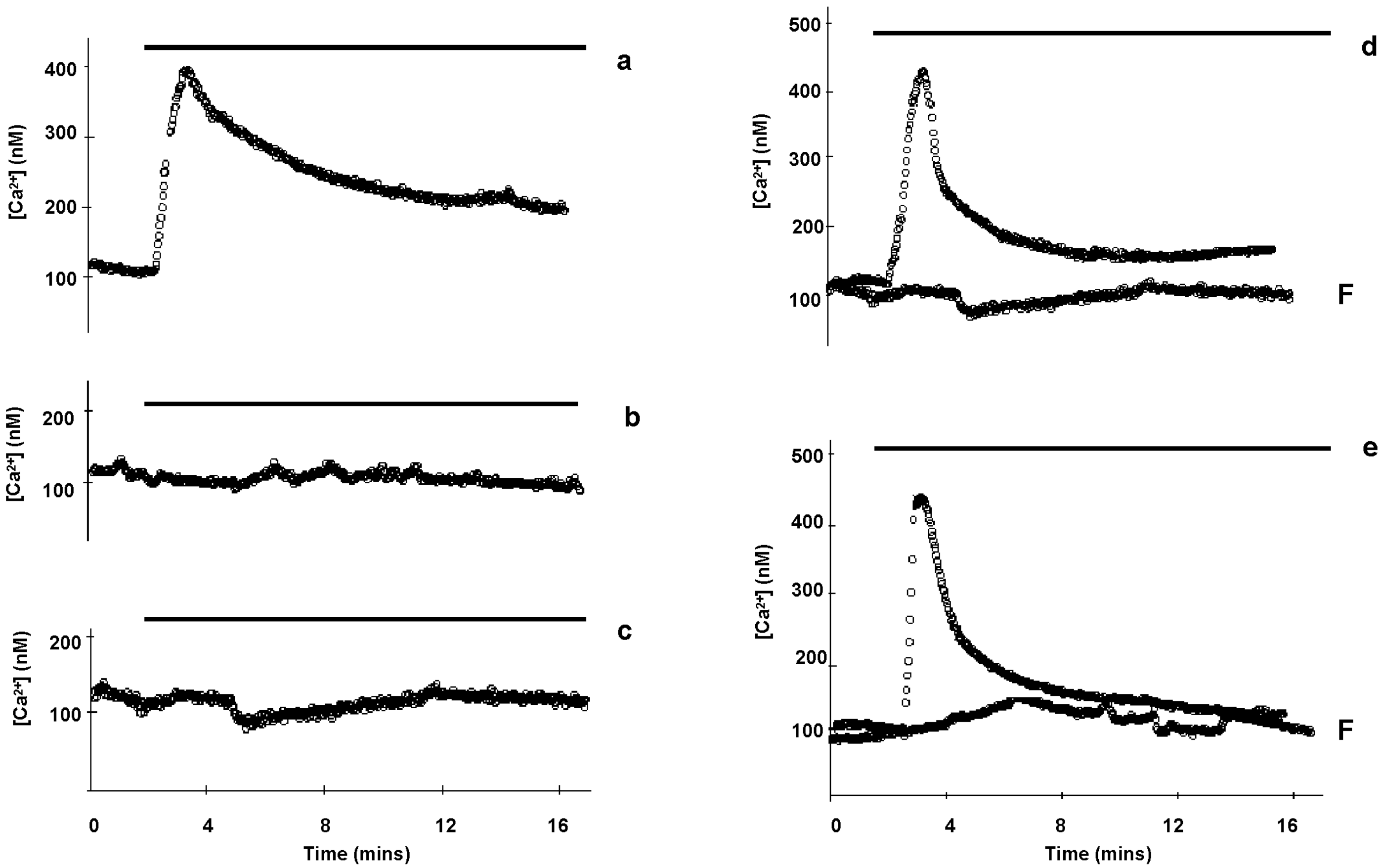

FIG. 3. Inhibition of the calcium response. The left curves show the effect of **a**) 1000 nM cyproterone acetate, **b**) 1000 nM hydroxyflutamide, and **c**) 1000 nM finasteride (F) on the response of rat Sertoli cells to 10 nM testosterone (present during the time indicated by the black bar). The curves on the right show the inhibiting effect of 1000 nM F during stimulation of Sertoli cells with **d**) 10 nM dihydrotestosterone and **e**) 10 nM R1881. Cells were preincubated for 20 min with inhibitors, which also remained present in the superfusion medium during the stimulation phase. Data are representative of at least six individual experiments.

Steroid Specificity

The specificity of the response system in the three cell types was tested by challenging the cells with various compounds at a concentration of 1000 nM and measuring the intracellular calcium levels for a period of 15 min. The androstane-derived compounds epitestosterone, 5β-dihydrotestosterone, androstenedione, 11β-hydroxyandrostenedione, dehydroepiandrosterone, 5α-androst-16en-3α-ol, 5β-androstan-3β-ol-17–one, 5α-androstane-3,17-dione, 5α-androst-3-one, 5α-androst-16-en-3-one, and 5α-androst-16-en-3β-ol did not stimulate calcium signaling. Similarly, progesterone and estradiol-17β; two antiandrogens, cyproterone acetate (CPA) and HF; and the inhibitor of 5α-steroid reductase, finasteride, had no effect on calcium levels.

Inhibition of Response

Inhibitory effects of various compounds were investigated by preincubating the cells with 1000 nM HF or CPA for 20 min prior to perfusion with 10 nM testosterone. The same responses were observed in the three cell types: HF completely blocked the androgen-induced calcium increases, whereas CPA did not interfere with the calcium signaling system. Finasteride, a specific inhibitor of 5α-steroid reductase [45], inhibited the response induced by 10 nM testosterone, R1881, and DHT when present at 1000 and 100 nM, but not at 10 nM (Fig. 3, Table 2).

Interactions of Androgens with the Cell Surface

To determine whether testosterone could induce a calcium response directly at the cell surface, testosterone covalently bound to BSA (T:BSA), which cannot enter the cells, was used. Figure 4, a and b, shows that the calcium response to 10 nM testosterone in Sertoli cells is the same for unbound testosterone and for testosterone covalently bound to albumin. No calcium response was found to unconjugated albumin. When Sertoli cells and LNCaP cells were perfused with T:BSA conjugated to FITC, an increase in fluorescence intensity was seen when T:BSA-FITC was in the perfusion chamber, but the fluorescence disappeared completely after washing out of the conjugate (Fig. 4c). BSA conjugated to FITC but without testosterone showed the same result (Fig. 4d), indicating that the presence of testosterone has no effect on the retention of the albumin conjugate on the plasma membrane. It was not possible to evaluate the biological activity of the FITC-labeled T:BSA because the FITC fluorescence made it impossible to measure the fluorescence of the calcium dyes. Thus, transient and reversible interactions between the androgen conjugate and the plasma membrane appear sufficient for generating at least the transient response.

We have also investigated interactions between unconjugated steroids and the plasma membrane of intact LNCaP cells by using tritiated R1881 and classical conditions for binding studies in intact cells. Because CPA can bind to

TABLE 2. Effect of inhibitors on intracellular calcium concentrations stimulated by 10 nM testosterone in rat Sertoli cells and human prostate cancer cells.

Response	Sertoli cells		LNCaP cells		PC3 cells	
	Basal[a] Ca^{2+} (nM)	Peak[b] Ca^{2+} (nM)	Basal Ca^{2+} (nM)	Peak Ca^{2+} (nM)	Basal Ca^{2+} (nM)	Peak Ca^{2+} (nM)
Control	138 ± 17[c]	431 ± 9[c]	144 ± 14	421 ± 19[d]	142 ± 19	432 ± 6[d]
Hydroxyflutamide (1000 nM)	144 ± 12	138 ± 7	139 ± 11	137 ± 7	147 ± 9	144 ± 11
Cyproterone acetate (1000 nM)	120 ± 16	430 ± 12[d]	132 ± 16	416 ± 11[d]	143 ± 7	428 ± 14[d]
Finasteride (1000 nM)	138 ± 13	141 ± 9	142 ± 11	144 ± 12	148 ± 10	146 ± 7

[a] Basal Ca^{2+} levels were recorded before addition of testosterone.
[b] Peak Ca^{2+} levels were recorded 2 min after testosterone addition.
[c] Data represent mean ± SEM of four individual experiments performed in triplicate.
[d] $P < 0.0005$, when compared with controls.

the nuclear androgen receptor but does not interfere with the calcium signaling pathway, it was used to suppress binding of R1881 to the nuclear receptor without influencing the membrane system. In the presence of 1000 nM CPA, specific binding of ^{3}H R1881 after 1 h incubation could not be demonstrated. This was because residual binding of 5 nM ^{3}H R1881 after 1 h incubation and 15 rapid washings with medium containing 1000 nM CPA could not be discriminated from nonspecific ^{3}H R1881 binding in the presence of 1000 nM unlabeled R1881. However, specific nuclear binding of R1881 was 10–20 times higher than nonspecific binding after equilibration of the cells with 5 nM ^{3}H R1881 for 1 h in the absence of CPA or unlabeled R1881 at either 4 or 37°C. With immunocytochemical techniques the presence of the nuclear receptor could be shown in the LNCaP cells and Sertoli cells, but not in the PC3 cells (data not shown).

Aspects of Calcium Signaling

To study whether the androgen-induced calcium increases are due to an extracellular influx of calcium or to calcium mobilization from intracellular stores, experiments were carried out without calcium in the medium. When 5 mM EGTA was added to the medium, the basal intracellular calcium level was reduced and addition of androgens did not induce an increase in calcium levels (Fig. 5b, Table 3). Following preincubation with a voltage-gated calcium channel blocker, verapamil (100 μM), for 20 min, only a small increase in calcium levels was observed after androgen addition (Fig. 5c, Table 3). These results show that calcium channels in the plasma membrane play an important role in calcium signaling.

Effects of Pulse Exposures to Testosterone

Because the binding studies showed that androgens interact reversibly with the plasma membrane, we have used the perfusion system to investigate the calcium response after transient exposure (150 sec) to androgens. Under these conditions, the persistent elevation of calcium after the transient peak was only 5.21% ± 1.33, for 10 nM testosterone, and 5.65% ± 1.91, for 100 nM testosterone (n = 6), lower than during continuous exposure to testosterone. The similarities in the response pattern between continuous and pulse exposure is an indication that the response is mainly determined by the initial changes in steroid concentrations. We therefore tested the response after two or three brief 30-sec pulses of 10 nM testosterone (Fig. 6, a and b), and found that the effect of the second or third pulse was less than the initial response. On one hand, the duration of the response after a 30-sec pulse of 10 nM testosterone (Fig. 6d) is transient, and calcium levels return to basal levels within approximately 30 min; on the other hand, a long-term response lasting for at least 50 min was obtained after a 30-sec stimulation with 100 nM testosterone (Fig. 6c).

Gap Junctional Communication

At least part of the calcium signaling pathways are coupled to phosphorylation cascades. Because GJIC can be modulated via phosphorylation, we have investigated whether androgens can rapidly affect GJIC. GJIC was measured using scrape loading-dye transfer in Sertoli cells because it is known that Sertoli cells communicate with each other via gap junctions [46]. Limited dye transfer was observed between Sertoli cells in the control situation (dye transfer 5.92% ± 0.36 of the area). The dye transfer was markedly increased 15 min after addition of testosterone to the cells (dye transfer 29.59% ± 1.06 of the area, n = 4, $P < 0.0005$), indicating an increase in GJIC (Fig. 7). A similar response to testosterone (dye transfer 28.31% ± 0.62 of the area, n = 4, $P < 0.0005$) was observed in the presence of cycloheximide (100 μM), which completely inhibits protein synthesis. LNCaP cells and PC3 cells showed no GJIC, indicating the specificity of the method.

DISCUSSION

The majority of androgen effects are mediated by the nuclear androgen receptor that is already maximally stimulated when free steroid concentrations in the target cells are 1 nM or lower [6]. It is difficult to envisage how these receptors can respond to higher androgen concentrations when they are already saturated. As yet there is no evidence that androgen receptors can produce specific signals depending on the degree of oversaturation. Nevertheless, biological effects at such high concentrations of androgens have been described [1–6, 44]. Thus, a low-affinity sensory system must operate to signal the changes in androgen concentration at these relatively high concentrations [7]. Many so-called nongenomic cell surface effects of steroids, recently described in the literature [8–26], possess this property and this alternative low-affinity sensory system could operate in conjunction with the nuclear high-affinity receptor [1, 3–5]. The results of the present investigation confirm that alternative androgen actions can take place at the plasma membrane level and that there is not only a response to high androgen levels up to 100 nM, but also to androgen concentrations of 1 pM. Moreover, they show a steroid specificity that has certain similarities with the nuclear receptor, but which also shows clear differences, especially with respect to effects of antiandrogens and finasteride.

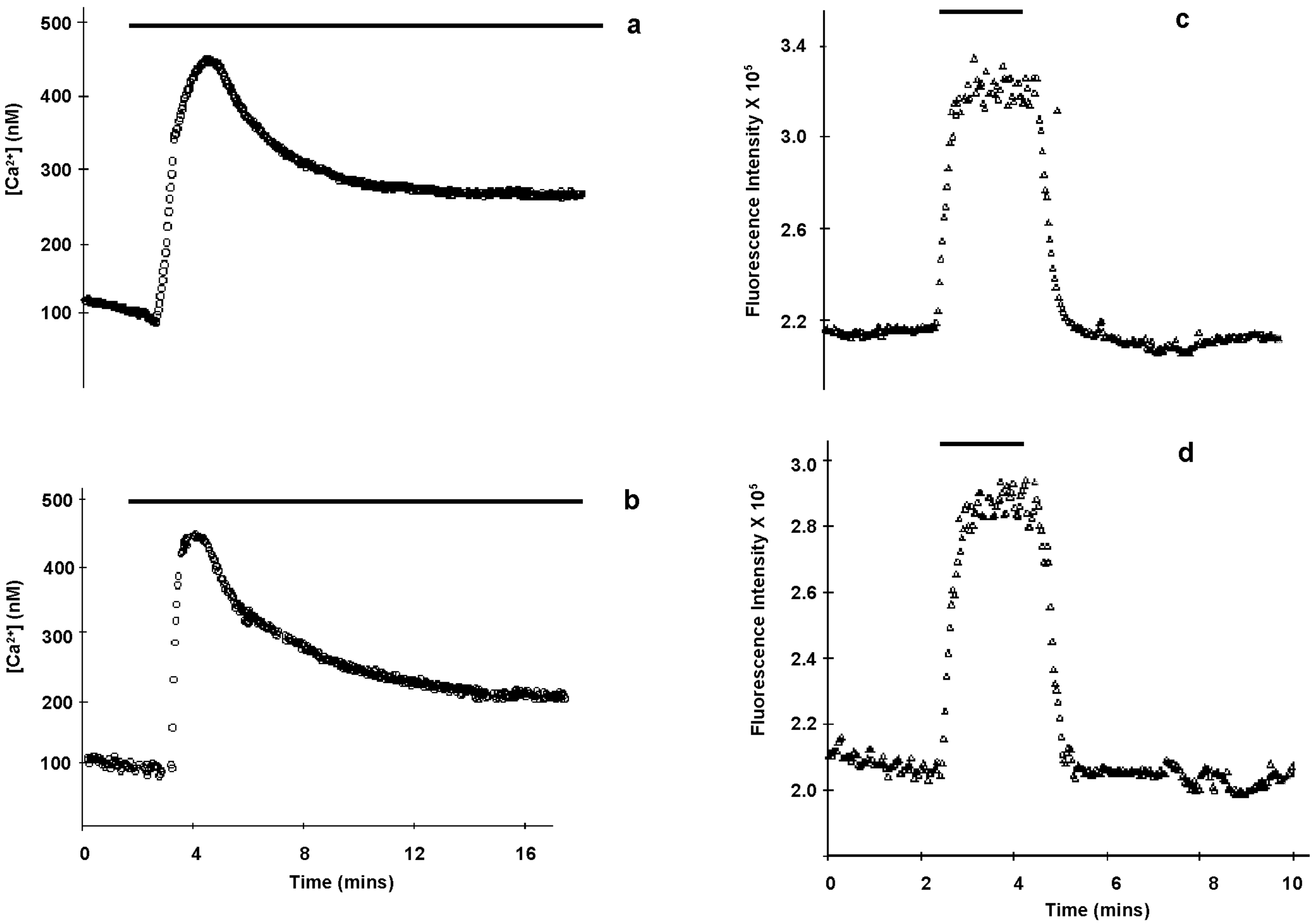

FIG. 4. Interactions between androgens and cell surface. The left curves show intracellular calcium levels in LNCaP cells before and during superfusion (indicated by the black bar) with **a**) 10 nM testosterone and **b**) 10 nM testosterone covalently bound to BSA. The right curves show the fluorescence intensity of LNCaP cells before, during, and after superfusion for 150 sec (black bar) with **c**) testosterone covalently bound to BSA-FITC and **d**) the BSA-FITC without testosterone. Similar results were obtained in two other experiments with LNCaP cells and three with Sertoli cells.

Steroid Specificity

The biologically active ligands, testosterone, DHT, and R1881, which can act via the nuclear receptor, were also active in rapid calcium signaling. Other steroids such as estradiol-17β, progesterone, 17α-testosterone, 5β-dihydrotestosterone, androstenedione, 11β-hydroxyandrostenedione, 5α-androst-16en-3α-ol, dehydroepiandrosterone, 5α-androstane-3,17-dione, 5α-androst-3-one, 5α-androst-16-en-3-one, 5α-androst-16-en-3β-ol, HF, CPA, and finasteride were not active even when tested at concentrations of 1000 nM. The testosterone-, R1881-, and DHT-induced calcium responses could be inhibited by the androgen antagonist HF; however, in contrast to effects on the nuclear receptor, the testosterone-, R1881-, and DHT-activated calcium transients were not inhibited by CPA but could be inhibited by finasteride, which is a specific inhibitor of 5α-steroid reductase [45].

The inhibition of the testosterone-induced effects by finasteride could, in theory, be explained by an inhibition of a very active 5α-reductase. However, R1881 is a metabolically stable androgen, testosterone is covalently bound to albumin, and DHT cannot be activated by intracellular 5α-reductase. Thus, it appears unlikely that the action of finasteride is due to inhibition of 5α-reductase activity. A more likely explanation is that finasteride interacts with the androgen binding site of the receptor at the cell membrane. The results with LNCaP cells also show that the binding site of the membrane system differs from the receptor in the nucleus because CPA, HF, progesterone, and estradiol, which can stimulate cell growth due to a mutation in the classical androgen receptor in this cell line [6], do not activate the receptor in the membrane.

Rapid effects of various androgens on LNCaP cells have been reported previously by Steinsapir et al. [20]. Effects of testosterone and DHT on calcium signaling in freshly isolated Sertoli cells have been reported by Gorczynska and Handelsman [26], but the lowest dose to show a response was 300 nM. Enzymatic and mechanical damage during the isolation procedure of the cells may have caused this low sensitivity because it has been shown that trypsin treatment reduces the membrane binding of monoclonal antibodies and cellular responses to glucocorticoids in lymphoma cells [27]. Gorczynska and Handelsman described that HF and finasteride blocked the effect of testosterone, whereas finasteride could not affect the response to DHT, leading to the conclusion that 5α-reduction of testosterone is necessary for the effect. Our data do not support this interpretation. Audy et al. [47] reported calcium fluxes in LNCaP cells induced by estradiol (100 pM–100 nM) and 10 nM DHT within 6 min. Flutamide completely inhibited the an-

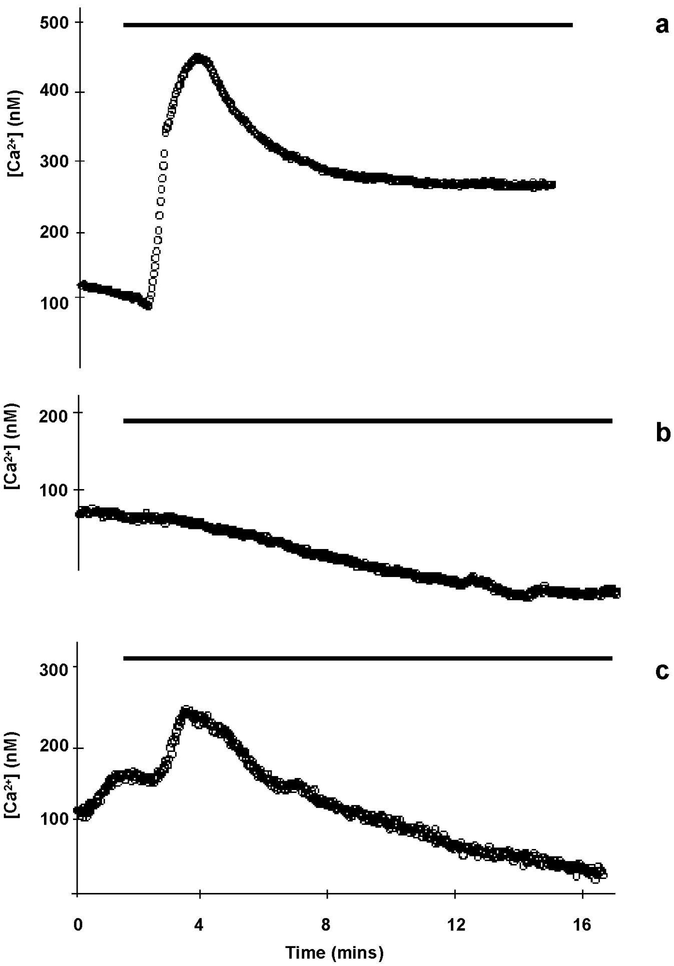

FIG. 5. Regulation of calcium response. Intracellular calcium response in rat Sertoli cells: effect of **a**) 10 nM testosterone, inhibition of response after preincubation for 20 min, and superfusion with **b**) 5 mM EGTA and **c**) 100 μM verapamil.

drogen response but had no effect on the estradiol response. Their data suggest that separate binding sites on the plasma membrane mediate the responses for the different sex steroids. Our responses showed completely different kinetics and steroid specificity without an effect of estradiol. Different LNCaP sublines could explain these discrepancies.

More than One Receptor

It is difficult to envisage how one binding site can sense and transmit information from a range of androgen concentrations that spans 5 orders of magnitude. A receptor clustering model has recently been proposed for chemotactic receptors on *Escherichia coli* bacteria to explain that one receptor for aspartate can sense gradients of attractants over 5 orders of magnitude [48]; however, there is no evidence that such a system operates in cells other than bacteria. Our calcium response data are compatible with a two-membrane receptor model in spermatozoa for progesterone, as shown by Luconi et al. [49]. Similar to their observations in spermatozoa with progesterone, we observed in three different cell types comparable biphasic dose-response curves generated by testosterone or R1881, but our responses occurred at approximately 1000-fold lower concentrations (pM and nM, respectively) when compared to progesterone. Similar to the restricted action of derivatives from progesterone on spermatozoa, DHT could activate only the low-affinity site of the responsive membrane system in our cells. It is unlikely that metabolism of DHT caused the absence of low-dose effects because under the perfusion conditions, the extracellular concentration of DHT is kept constant. Moreover, if metabolism had occurred, the entire dose-response curve would have been shifted to the right.

At the present time there is no structural information of the receptor for androgens in the membrane. Results from recent experiments show that classical nuclear receptors (perhaps modified), especially estrogen receptors, can also activate the mitogen-activated protein kinase pathway [28]. It has also been shown that a small fraction (4%) of the nuclear receptor can associate with the plasma membrane and that these membrane-associated receptors also bind the same ligands as the nuclear receptor with a similar high affinity. They can also activate signal transduction pathways via G proteins, although with a different steroid specificity, when compared with the nuclear receptor [29]. For the androgen receptor, there is also evidence for alternative processing. Immunoreactivity of androgen-receptor proteins have been shown in T cells but no androgen binding activity could be shown [23, 24]; however, there was no evidence that such alternatively processed products were present in the plasma membrane of T cells. The membrane receptor in our cells could, in theory, also originate from alternative processing of the nuclear receptor, but the many similarities in the response characteristics independent of the nuclear receptor status; no receptors in PC3 cells, mutated receptors with very different steroid specificity in LNCaP cells, and normal receptors in Sertoli cells, make this possibility very unlikely. Moreover, the interactions between androgens and receptors on the plasma membrane and in the nucleus are so different that, with classical binding techniques (including several washing steps), we could not detect any specific retention of R1881 to the plasma membrane of LNCaP cells, whereas nuclear binding was

TABLE 3. Effects of EGTA and Verapamil on intracellular calcium concentrations stimulated by 10 nM testosterone in rat Sertoli cells and human prostate cancer cells.

	Sertoli cells		LNCaP cells		PC3 cells	
Response	Basal[a] Ca^{2+} (nM)	Peak[b] Ca^{2+} (nM)	Basal Ca^{2+} (nM)	Peak Ca^{2+} (nM)	Basal Ca^{2+} (nM)	Peak Ca^{2+} (nM)
Control	138 ± 17[c]	431 ± 9[d]	144 ± 14	421 ± 19[d]	142 ± 19	432 ± 6[d]
EGTA (5 mM)	65 ± 16	43 ± 12	51 ± 14	32 ± 16	68 ± 8	59 ± 6
Verapamil (100 μM)	144 ± 11	259 ± 15[e]	141 ± 12	252 ± 6[e]	145 ± 9	234 ± 13[e]

[a] Basal Ca^{2+} levels were recorded before addition of testosterone.
[b] Peak Ca^{2+} levels were recorded 2 min after testosterone addition.
[c] Data represent mean ± SEM of four individual experiments performed in triplicate.
[d] $P < 0.0005$, when compared with controls.
[e] $P < 0.001$ when compared with controls.

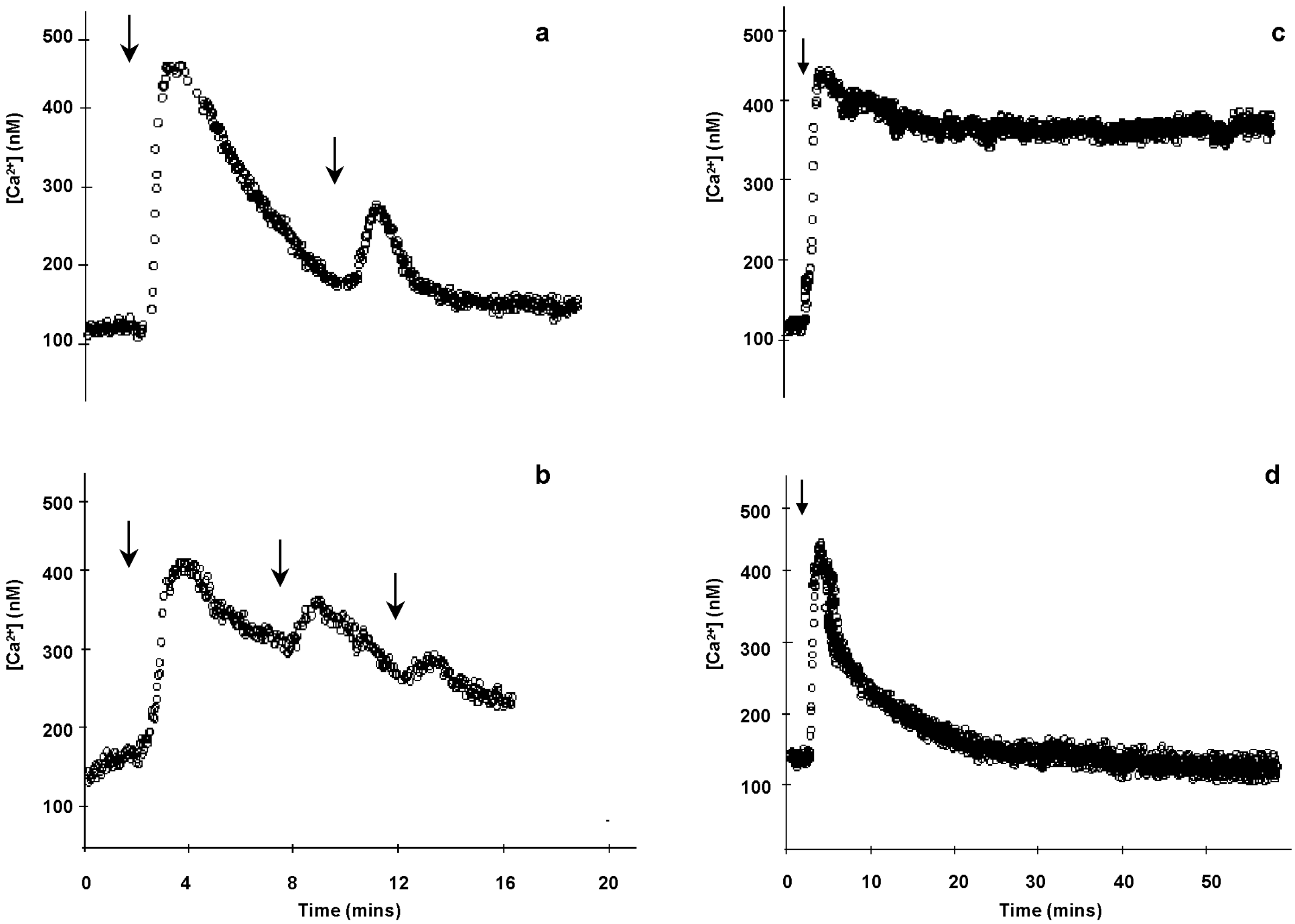

FIG. 6. Effects of pulse exposures to androgens. Intracellular calcium levels in rat Sertoli cells were recorded before, during, and after **a**) two and **b**) three consecutive 30-sec pulses of 10 nM testosterone (arrows). The right curves (**c** and **d**) show the effect of a single 30-sec pulse of **c**) 100 nM testosterone and **d**) 10 nM testosterone (arrow) over a 60-min instead of a 16-min period. Similar observations were made in four other experiments.

normal. A high dissociation rate constant for these membrane interactions could explain the poor retention of R1881.

As a result of these findings, we propose that proteins not coded by the mRNA for the nuclear receptor most probably mediate the calcium signaling.

Characteristics of Calcium Signaling

High calcium levels persisted after brief exposure (150 or 30 sec) to high concentrations of androgens when cells were perfused with medium without androgens for periods up to 45 min. Because androgens do not bind tightly to the membrane, this retention of the calcium response occurs without the presence of steroids on the membrane receptors. Thus, a brief exposure to androgens can induce a long-term response. All we can conclude from experiments with calcium channel blockers and zero calcium in the incubation medium is that external calcium is required for the calcium signaling, but we do not know how this activated state is initiated by the presence and maintained in the absence of androgens.

Lieberherr and Grosse [22] described dose-dependent effects of androgens in osteoblasts also greater than 5 orders of magnitude of androgen concentrations, but their dose response curve was bell-shaped. Their responses were also different from ours in other respects: osteoblasts responded within 5 and not 30 sec after administration of the androgen; testosterone and DHT gave similar responses; and the transient calcium response was not inhibited by the absence of extracellular calcium, whereas it was inhibited by inhibitors of phospholipase C. Together with our data, there are thus many different possibilities for activation of calcium transients by cell surface actions of androgens.

Effects on Gap Junctions

We have investigated changes in GJIC between Sertoli cells as a possible downstream event of the calcium signaling pathway because it is known that variations in second messengers such as Ca^{2+}, H^+, and cAMP can regulate the efficiency of the gap junctions via phosphorylation of the connexin proteins [50]. Androgens increased gap junctional efficiency between Sertoli cells within 15 min in the absence of protein synthesis. We have interpreted this gap junctional regulation as a nongenomic, functional response of the Sertoli cell to testosterone, which could be mediated via a calcium signal. Pluciennik et al. [51] have shown an increase in GJIC following incubation of rat Sertoli cells with FSH. The same authors also reported an interruption of the junctions after 15 min of exposure to 1–25 μM testosterone propionate or estradiol-17β propionate [52, 53].

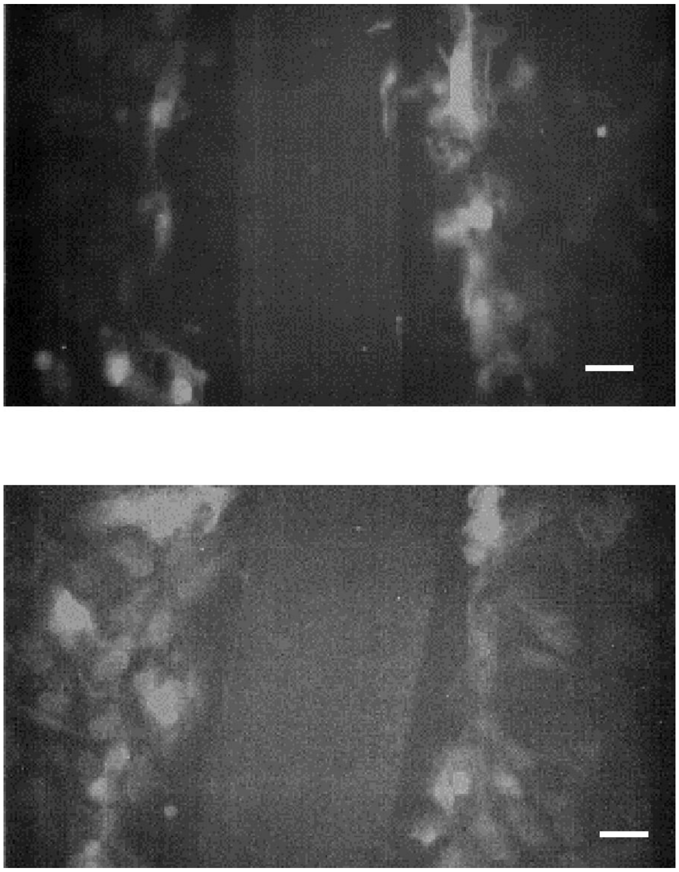

FIG. 7. Gap junctional intercellular communication (GJIC). Fluorescence dye transfer through gap junctions in (upper panel) control and (lower panel) testosterone-treated rat Sertoli cells. Cells were incubated with 10 nM testosterone for 15 min and subsequently scrape-loaded with lucifer yellow dye. Dye transfer is a measure of GJIC. Micrographs are representative of six individual experiments. Bar = 20 μm.

These effects were observed only with high concentrations of steroid esters in the absence of changes in intracellular calcium concentrations, suggesting direct effects of the steroid esters on membrane properties. Thus, there appear to be different modes for regulation of GJIC between Sertoli cells, with androgen-induced calcium signaling pathways as one of the possibilities.

Biological Relevance of Androgen-Induced Calcium Signaling

An important question is whether these androgen-activated signaling pathways can activate specific cellular functions or whether they merge with pathways already under the control of protein hormones or growth factors. As stated in the *Introduction,* Sertoli cells require high local androgen levels for proper functioning. It is possible that the persistent high calcium levels, which are generated only by high levels of androgens and not by EGF or FSH, are a reflection of such a specific pathway. Such a specific calcium pathway could explain why significant stimulation of ABP secretion by a combination of Sertoli and peritubular myoid cells occurs only when testosterone concentrations are higher than 4 nM [5] and not between 0.1–1 nM, which is the normal sensitivity for androgen-responsive cells [6].

In LNCaP cells, low levels of androgens stimulate cell growth, whereas concentrations higher than 1 nM (above the affinity constant of the receptor), inhibit cell growth, further stimulate the secretion of prostate specific antigen, and increase the percentage of androgen-receptor-positive cells [54]. Other investigators [6, 44, 55] using LNCaP cells have also observed growth-inhibitory effects of R1881 and DHT when present at concentrations higher than 1 nM, whereas HF, CPA, progesterone, and estradiol-17β all stimulate cell growth even at 100 nM. The androgen receptor is essential for this growth inhibition because androgen-dependent growth inhibition also occurs in MCF-7 cells after transfection with androgen receptors, and is maximal at concentrations of R1881 below 1 nM [56]. It is puzzling that inhibition of cell growth in LNCaP cells requires higher concentrations than 1 nM R1881, testosterone, or DHT and that other ligands that stimulate the mutated androgen receptor do not induce growth inhibition. Because we have shown that only R1881, testosterone, and DHT stimulate intracellular calcium in LNCaP cells, we hypothesize that high intracellular calcium somehow plays a role in the androgen receptor-mediated growth inhibition. The growth response of muscles to pharmacological doses of androgens (drug abuse), is also difficult to explain with the properties of the high-affinity nuclear receptor alone [57, 58]. This hypertrophy of muscle cells may thus also require a complementary low-affinity response system in addition to the high-affinity androgen receptors in the nucleus.

It is known that membrane receptors for proteins can modulate different nuclear steroid receptors, they can even activate the steroid receptors without the presence of ligand [59]. The importance of such external regulatory networks for the outcome of steroid hormone action in the nucleus was recently stressed by O'Malley et al. [60], when they proposed that membrane transduction pathways, activated by growth factors, which interact with the nuclear receptor via intracellular phosphorylation cascades, may ''set the nuclear receptor thermostat'' for responses to steroids. In a similar fashion, the androgen-induced calcium signaling pathway can also influence the functional properties of the nuclear androgen receptor. To investigate this possibility for fine-tuning of the androgen receptor, selective agonists and more knowledge on the structure and function of the putative membrane receptor or receptors is necessary.

NOTE ADDED IN PROOF

Important new information on variability of hormone induced calcium signaling can be found in a multi-author commentary: ''Calcium confusion: is the variability in calcium response by Sertoli cells to specific hormones meaningful or simply redundant?'' by FFG Rommerts, FM Lyng, E von Ledebur, L Quinlan, GR Jones, JB Warchol, M Stefanini, N Ravindranath, M Joffre. J Endocrinol; 2000.

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