© European Journal of Endocrinology 1996, 135: 367–73

17β-Estradiol stimulates a rapid Ca^{2+} influx in LNCaP human prostate cancer cells

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Audy MC, Vacher P, Dufy B. 17β-Estradiol stimulates a rapid Ca^{2+} influx in LNCaP human prostate cancer cells. Eur J Endocrinol 1996;135:367–73. ISSN 0804–4643

Prostate growth is known to be controlled by steroids such as androgens and estradiol. For this reason steroids (estradiol, adrenal androgens) or steroid inhibitors are commonly used as palliative treatments for prostate carcinoma. In view of the pivotal role played by Ca^{2+} ions in cell proliferation, we decided to investigate the effects of 17β-estradiol (E_2) on intracellular calcium concentration ($[Ca^{2+}]_i$) in a human prostate tumor cell line, LNCaP. In this study, we show that E_2 induced a dose-dependent (0.1–100 nmol/l) influx of Ca^{2+} in these cells. These effects occurred rapidly after the beginning of the ejection and were maintained in the presence of the hormone (plateau phase). Estradiol-induced Ca^{2+} influx was unaffected by the saturation of the androgen receptor with pure antiandrogen flutamide. The use of tamoxifen, an antiestrogen binding to nuclear receptors, or E_2 covalently linked to bovine serum albumin that cannot penetrate the cell membrane, did not block the ($[Ca^{2+}]_i$) response. Our results suggest the existence of E_2 binding sites at the plasma membrane surface of LNCaP cells, linked to calcium signalling and, more specifically, Ca^{2+} channels.

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Sex steroids (androgens and estrogens) are major regulators of prostate growth. For this reason, anti-androgen and estrogen therapies have been widely used as standard treatments for advanced prostate cancer, but the mechanisms by which they exert their effects are still unclear. Most of the known effects of steroid hormones are mediated by receptors in the cell nucleus that, upon ligand binding, act to modulate the transcription activity of the responsive cells. However, rapid steroid effects in various cell types, such as hepatocytes (1), granulosa cells (2), oocytes (3), spermatozoa (4), uterine smooth-muscle cells (5), intestine cells (6), osteoblasts (7) and prostate cells (8), suggest the possibility of alternative non-genomic action mechanisms, presumably at receptors on the cell surface.

The establishment of an androgen-sensitive human prostate cell line, LNCaP, has provided an in vitro model for prostate carcinoma cell study (9). Previous research had shown that estrogen- or progesterone-induced cell growth occurred in LNCaP cells, despite the absence of specific estrogen and progesterone receptors (10). A mutation allowing the binding of various steroids and antisteroids to the abnormal androgen receptor system of LNCaP has been described (11). However, more recently, 17β-estradiol (E_2) binding sites have been identified in both cytosol and nuclear fractions of LNCaP (12) and it has been shown that the proliferative action of E_2 was mediated by its "own" receptors (13).

Cell calcium metabolism is intimately involved in the regulation of many biological and biochemical activities. Data from several recent studies have shown that calcium plays a significant role in DNA synthesis and cell proliferation (14). The non-genomic action of steroids is receiving renewed interest. However, only little information is available yet about the early effects of steroids on intracellular calcium. Included in this information are recent reports showing increases in intracellular calcium concentration ($[Ca^{2+}]_i$) levels via Ca^{2+} influx in response to androgens in LNCaP cells (8) and osteoblasts (15), while increases in $[Ca^{2+}]_i$ levels in response to estrogen in granulosa cells occurred via intracellular Ca^{2+} mobilization (2).

The aim of this study was to investigate a possible effect of estrogen on $[Ca^{2+}]_i$ in LNCaP cells. 17β-Estradiol covalently coupled to high-molecular-weight molecules that cannot enter the cell (4) was also used to examine the possible involvement of a plasma membrane receptor. It was found that E_2 induced a rapid influx of Ca^{2+}, independently of androgen receptors.

Material and methods

Cell culture

LNCaP cells (passage 39) were maintained in RPMI 1640 medium (BioWhittaker Seromed, Strasbourg France) containing 10% fetal calf serum (Gibco, Grand Island, NY) at 37°C in a humidified atmosphere (5% CO_2/95% air). They were fed with fresh medium every 2 or 3 days for 8 days. The cells were seeded on 10-mm

round glass coverslips pretreated with polyornithine (5 μg/ml). Forty-eight hours before the experiments, the normal culture medium was replaced by RPMI 1640 red phenol-free with 10% charcoal–dextran-treated fetal calf serum.

Measurements of cytosolic free Ca^{2+} concentration $[Ca^{2+}]_i$

The experiments were performed using the fluorescent probe indo1 on the cell population as already described (16). Before the microspectrofluorimetric experiment, the nutrient medium was replaced by a modified Hank's solution containing 142.6 mmol/l NaCl, 5.6 mmol/l KCl, 2 mmol/l $CaCl_2$, 0.8 mmol/l $MgCl_2$, 5 mmol/l glucose and 10 mmol/l HEPES, buffered to pH 7.3 with NaOH. LNCaP seeded on 10-mm round coverslips were loaded with the fluorescent probe indo1 by exposure to 5 mmol/l indo1 AM (Calbiochem, Paris, France) in Hank's solution for 30 min at 37°C. The glass coverslide carrying the loaded cells was placed on a plastic holder in a quartz cuvette and $[Ca^{2+}]_i$ was estimated from indo1 fluorescence by the ratio method, using single wavelength excitation (350 nm) and dual wavelength emission (405 and 480 nm) in a Hitachi F2000 spectrofluorimeter. The background fluorescence of unloaded cells was subtracted from each measurement before the ratio determinations. The indo1 fluorescence response to the intracellular calcium concentration was calculated from the 405/480 nm ratio of fluorescence values as described by Grynkiewciz et al. (17). The dissociation constant for the indo1–Ca^{2+} complex was taken as 405 nmol/l. The values for R_{max} and R_{min} were calculated from measurements using 100 μmol/l digitonine and 10 mmol/l EGTA. Hormones and "anti-hormones" were added either alone or simultaneously according to the experimental protocol.

Test substances

17β-Estradiol (E_2), dihydrotestosterone (DHT), flutamide (2-methyl-*N*-(4-nitro-3-(trifluoromethyl)phenyl)propanamide, FLU) and tamoxifen ((Z)-1-(*p*-dimethylaminoethoxyphenyl)-1,2-diphenyl-1-butene, TX) were purchased from Sigma (St Louis, MO); stock solutions (10^{-2} mol/l in ethanol) were serially diluted in Hank's solution before use. We utilized E_2 covalently linked on the steroid nucleus via an *O*-carboxymethyloxime(CMO) to BSA (E_2 CMO BSA) that cannot enter the cell. The preparation of E_2 CMO BSA contained 30 mol E_2/mol BSA (Sigma St Louis, MO); a stock solution (0.1 mmol/l) was diluted in 0.1% BSA and 150 mmol/l NaCl. Free E_2 was removed from solutions as described by Blackmore et al. (4): 100 μl of E_2 CMO BSA (100 μmol/l), E_2 CMO (100 μmol/l) or E_2 (100 μmol/l) was incubated with 20 μl of charcoal (0.5 g/ml; BSA 4 mg/ml) at room temperature (4). They were administered in the spectrofluorimetric cuvette by extracellular pipette ejection.

Statistical analysis

Results were expressed as $[Ca^{2+}]_i \pm$ SEM or as the percentage±SEM increase. Statistical comparisons were performed by one-way analysis of variance using ANOVA and Fisher PLSD as post-tests. Student's *t*-test was used when appropriate, $p < 0.1$ was considered to be significant.

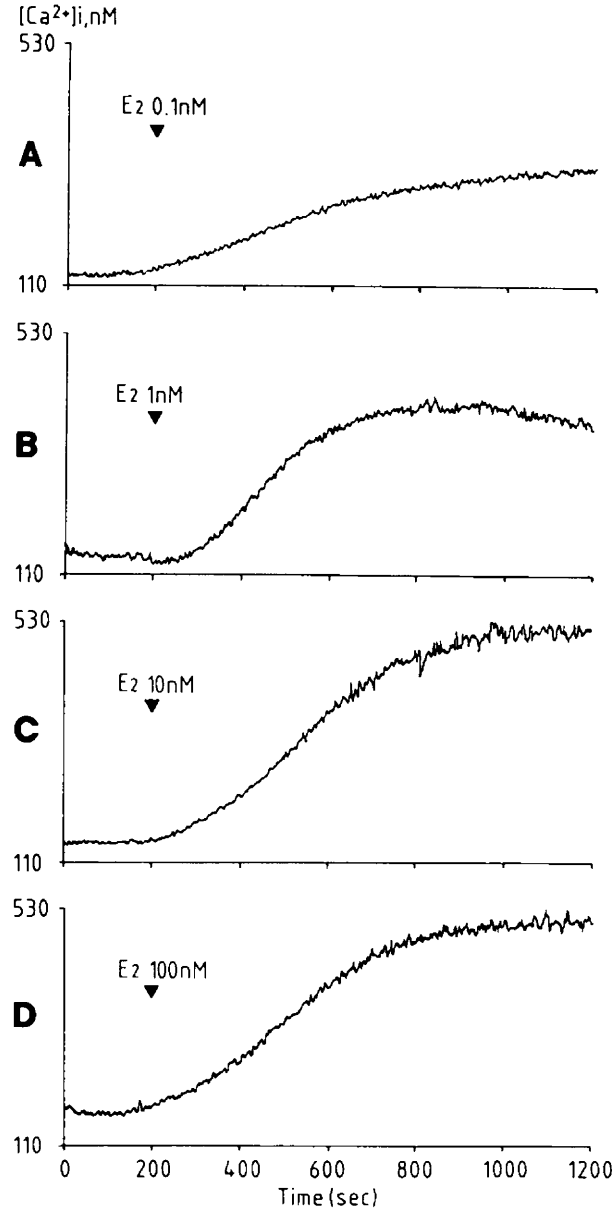

Fig. 1. Effects of long applications of E_2 on intracellular calcium concentrations $[Ca^{2+}]_i$ in LNCaP cells; $[Ca^{2+}]_i$ was measured in cell populations by microfluorimetry (dual wavelength emission technique) using indo1 as the Ca^{2+} fluorescent probe. The arrow indicates the beginning of injection. Estradiol was present in the bath until the end of the recording; 0.1 (A), 1 (B), 10 (C) and 100 (D) nmol/l E_2 induced a slow increase in $[Ca^{2+}]_i$.

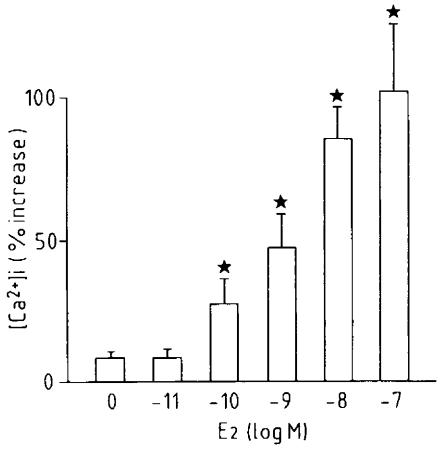

Fig. 2. Relationship between agonist doses and the percentage increase in intracellular calcium concentration ($[Ca^{2+}]_i$) (control being basal $[Ca^{2+}]_i$). Maximal plateau values of $[Ca^{2+}]_i \pm$ SEM were obtained between 1000 and 1200 s after administration of E_2 in the bath medium; *$p < 0.01$.

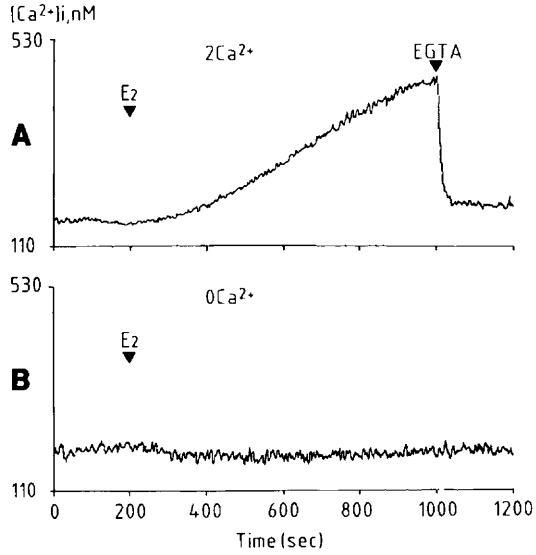

Fig. 3. Effect of an application of 10 nmol/l E_2 on the intracellular calcium concentration ($[Ca^{2+}]_i$) of LNCaP cells bathed in control medium (A) or a medium containing 0 mmol/l Ca^{2+} and 2 mmol/l EGTA (B). In the absence of extracellular free Ca^{2+}, the application of E_2 did not elicit any response. The arrow indicates the beginning of the injection.

Results

Effect of E_2 on $[Ca^{2+}]_i$

The resting $[Ca^{2+}]_i$ in LNCaP cells was 133 ± 6 nmol/l. No spontaneous Ca^{2+} oscillations were observed in these cells.

Application of E_2 rapidly elicited a dose-dependent $[Ca^{2+}]_i$ rise, which was maintained (plateau) as long as the steroid was present in the medium; E_2 was effective in the concentration range from 0.1–100 nmol/l (Fig. 1). A low concentration of E_2 (0.01 nmol/l) was unable to induce any significant $[Ca^{2+}]_i$ increase compared to the control ($8.5 \pm 2.5\%$ vs $8.3 \pm 2.1\%$ NS); from 0.1 to 100 nmol/l E_2 the maximal value of $[Ca^{2+}]_i$ increased significantly ($28.5 \pm 8.3\%$, $N = 4$, $p < 0.05$ for 0.1 nmol/l E_2; $47.0 \pm 12.2\%$, $N = 3$, $p < 0.01$ for 1 nmol E_2; $84.4 \pm 12.3\%$, $N = 11$, $p < 0.01$ for 10 nmol/l E_2; $103.8 \pm 29.2\%$, $N = 5$, $p < 0.01$ for 100 nmol/l E_2) (Fig. 2). A concentration as low as 10 nmol/l produced a maximal effect, with no further increase for concentrations up to 100 nmol/l E_2 ($p > 0.01$). A concentration of 10 nmol/l was therefore chosen to characterize the E_2-induced $[Ca^{2+}]_i$ in the other experiments. The half-maximal value ($63 \pm 12\%$ above control) was obtained 360 ± 40 s after the end of the ejection.

Role of extracellular and intracellular calcium in the E_2-induced $[Ca^{2+}]_i$ response

To determine the origin (Ca^{2+} influx or intracellular Ca^{2+} mobilization) of Ca^{2+} ions mobilized by E_2, an ejection of 10 mmol/l EGTA was applied during the E_2-induced $[Ca^{2+}]_i$ rise, in order to chelate Ca^{2+} ions contained in the extracellular medium. This resulted in complete blockade of the response (Fig. 3A). Similarly, application of E_2 to cells bathed in Ca^{2+}-deprived medium did not elicit any $[Ca^{2+}]_i$ response (Fig. 3B). These results suggested that the effects of E_2 were exclusively due to the stimulation of Ca^{2+} entry through Ca^{2+} channels.

Estrogen binding sites in LNCaP cells

As estrogen binding sites have been described in LNCaP cells (18), we investigated whether the effects of E_2 on $[Ca^{2+}]_i$ were due to the binding of the steroid to its own receptor or to a mutated androgen receptor. For this purpose LNCaP cells were pretreated with a pure antiandrogen, flutamide (FLU 10 nmol/l for 20 min), in order to compete with the androgen receptor. Although FLU pretreatment completely abolished the DHT-induced $[Ca^{2+}]_i$ response (Fig. 4D), it did not affect the E_2-induced $[Ca^{2+}]_i$ increase ($93.7 \pm 17.1\%$, $N = 7$ vs $84.4 \pm 12.3\%$, $N = 11$; NS) (Fig. 4B). Tamoxifen, described as an antiestrogen acting on intracellular receptors, was used to block the estrogen receptor. Tamoxifen alone (100 nmol/l or 1 μmol/l) did not affect $[Ca^{2+}]_i$. Pretreatment with tamoxifen (100 nmol/l or 1 μmol/l for 20 min) did not block the E_2-induced Ca^{2+} response (Fig. 5), suggesting that E_2 nuclear receptors were not involved. To confirm this hypothesis, we used immobilized E_2 coupled to high-molecular-weight BSA that cannot penetrate the plasma membrane. Free E_2 was suppressed by charcoal treatment. The efficiency of this treatment was confirmed by applying a charcoal-pretreated solution of 10 nmol/l E_2 to the cells. This solution was unable to increase $[Ca^{2+}]_i$ (E_2 $11 \pm 1\%$,

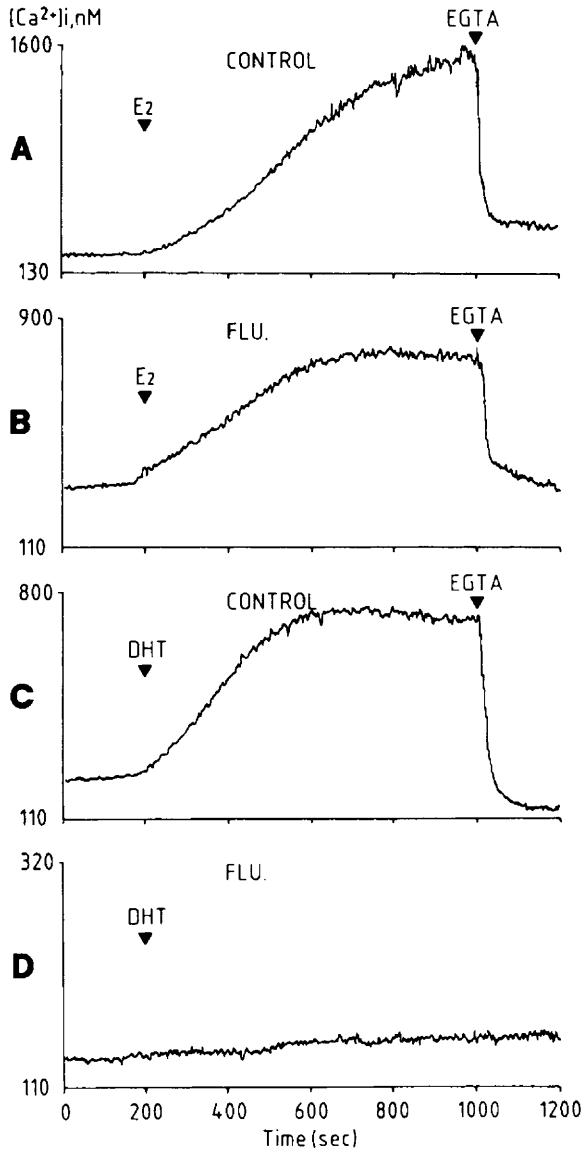

Fig. 4. Intracellular calcium levels in LNCaP cells before and after the administration of 10 nmol/l E_2 (A and B) or of 10 nmol/l dihydrotestosterone (DHT) (C and D) in LNCaP cells pretreated (B and D) or not (A and C) with 10 nmol/l flutamide (FLU) for 20 min. The antiandrogen FLU was present throughout the entire recording period. Pretreatment with the antiandrogen FLU did not modify the response to E_2, whereas the response to DHT was completely blocked.

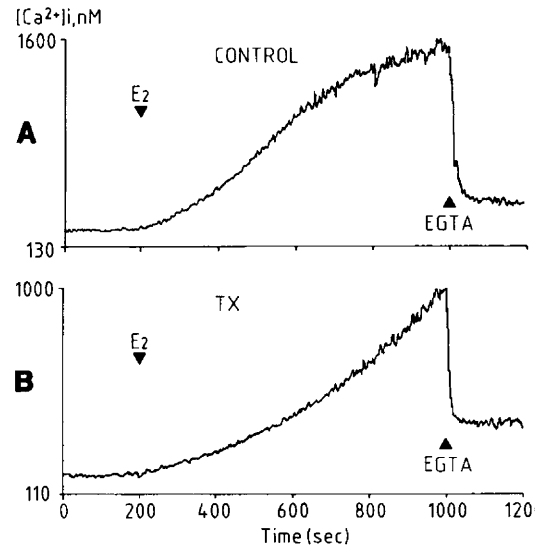

Fig. 5. Intracellular calcium levels in LNCaP cells measured after the administration of 10 nmol/l E_2 to LNCaP cells pretreated (B) or not (A) with 100 nmol/l tamoxifen for 20 min before the addition of E_2. The antiestrogen tamoxifen was present throughout the entire recording period. Pretreatment with the antiestrogen tamoxifen did not block the response to E_2, although it modified the kinetics of the E_2-induced Ca^{2+} increase

N = 3 vs HBSS 8.3 ± 2%, N = 3; NS) (Fig. 6C). The 10 nmol/l E_2 CMO elicited a rise in $[Ca^{2+}]_i$ but was less potent than 10 nmol/l E_2 (E_2 CMO 60.0 ± 9.2%, N = 3 vs 84.5 ± 15.3%, N = 3; $p > 0.01$) (Fig. 6A). The 10 nmol/l E2 CMO BSA treated with charcoal induced a $[Ca^{2+}]_i$ response showing the same profile as the control (E_2 CMO BSA charcoal-treated 66.5 ± 26.7%, N = 4 vs E_2 CMO 60.0 ± 9.2%, N = 3; NS) (Fig. 6B); BSA alone had no effect (Fig. 6D).

Discussion

It is widely recognized that LNCaP cells are a good model for in vitro studies of human prostate cancer cells (9). Previous reports have demonstrated that LNCaP cells possess a mutant androgen receptor able to bind not only androgens but also antiandrogens, estrogens and progestins (11, 18). Androgens, mainly DHT (19), represent the main determinant in induction, growth and maintenance of the LNCaP cell line (10, 19–22). However, estrogen has been described as playing a role in growth control, both in androgen-sensitive (LNCaP) (13) and insensitive (PC3) (23) prostate cancer cell lines. For this reason, oral androgen treatment was rapidly associated with the administration of GnRH (24, 25) or E_2 (26, 27) to enhance the cytotoxic effect.

The existence of estrogen receptors in prostate cells has long been controversial. However, multiple binding sites have been described recently in prostate cells and benign prostatic hyperplasia (12, 28). In LNCaP cells, it has been reported recently that the biological response to E_2 is clearly mediated by its own receptors (13).

Numerous data are available concerning the genomic molecular action mechanism of steroids. The receptors appear to be predominantly nuclear (29), with a hormone–receptor complex regulating gene expression. On the other hand, the rapid response evoked by applying steroids to target cells is puzzling. In Sertoli cells, androgen causes a rapid increase in $[Ca^{2+}]_i$ (30); in LNCaP cells an influx of Ca^{2+} through L-type calcium channels is induced by androgen (8); in chicken and pig

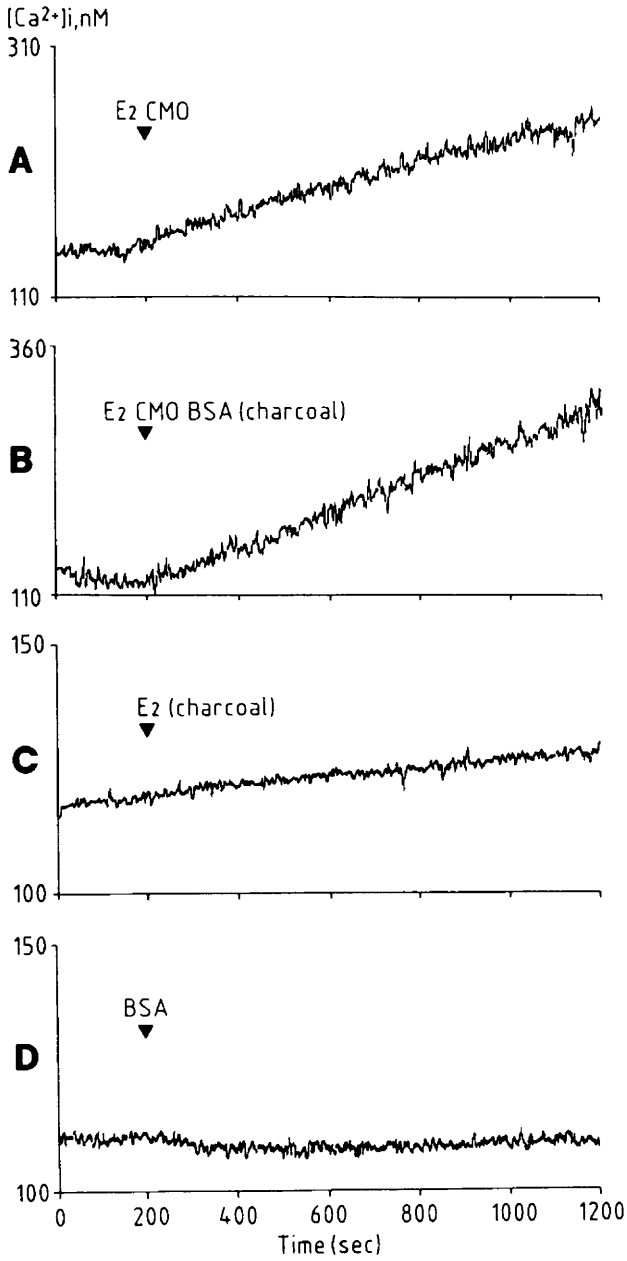

Fig. 6. Intracellular calcium concentration ($[Ca^{2+}]_i$) levels in LNCaP cells after the administration of E_2 covalently linked on the steroid nucleus via an O-carboxymethyloxime (CMO) to BSA (E_2 CMO BSA); a solution of E_2 CMO was used as the control. The $[Ca^{2+}]_i$ was measured after the administration of E_2 CMO (A), charcoal treated E_2 CMO BSA (B), charcoal-treated E_2 (C) or BSA (D). Like E_2, 10 nmol/l E_2 CMO induced an increase in $[Ca^{2+}]_i$. Although 10 nmol/l E_2 CMO BSA could not penetrate the cells, it still produced an increase in $[Ca^{2+}]_i$. BSA alone had no effect on $[Ca^{2+}]_i$.

ovarian granulosa cells E_2 triggers a rapid release of intracellular Ca^{2+} (2). Here, we show the rapid effects of E_2 on $[Ca^{2+}]_i$ in LNCaP cells. 17β-Estradiol triggered a rapid and sustained increase in $[Ca^{2+}]_i$ due to Ca^{2+} influx. The fact that the prompt E_2-induced $[Ca^{2+}]_i$ was not affected by incubating the cells with a pure antiandrogen, flutamide, which binds to androgen receptors and suppresses the DHT-induced Ca^{2+} rise, suggests the existence of specific E_2 receptors (13). It has been shown that the failure of tamoxifen to prevent the E_2-induced $[Ca^{2+}]_i$ rise is due to E_2 receptors other than the conventional nuclear receptors (31). In LNCaP cells the rapid response and the inability of tamoxifen to block the response also suggests a non-genomic action triggered at cell membrane level. Rapid effects of steroid hormones at the cell membrane level have been described also in neuronal tissue (32). It has been reported that immobilized ligands, coupled to high-molecular weight molecules such as BSA, do not enter the plasma membrane (4). When 10 nmol/l E_2 CMO BSA, treated in a charcoal bath to suppress any free E_2, was applied to LNCaP cells, an E_2-induced $[Ca^{2+}]_i$ rise was still observed, confirming a binding at the surface of the plasma membrane. The response to E_2 in LNCaP cells is quick (measured in terms of minutes), but less so compared to the E_2 stimulation of Ca^{2+} mobilization (measured in terms of seconds) as reported in granulosa cells (2). Moreover, the response of $[Ca^{2+}]_i$ after E_2 BSA application was weaker. Also, the kinetics of the E_2-induced Ca^{2+} rise appeared slightly modified in the presence of tamoxifen. Thus, the participation of E_2 nuclear receptor binding sites with an E_2-induced membrane Ca^{2+} influx cannot be completely excluded. Receptor-activated $[Ca^{2+}]_i$ variations have been reported in a large number of excitable and non-excitable cells (33–36). In osteoblasts, an androgen-induced rise in $[Ca^{2+}]_i$ has been reported (15). This increase is apparently mediated through membrane receptors coupled to phospholipase C and G proteins. Both Ca^{2+} influx and Ca^{2+} mobilization from the endoplasmic reticulum contributed to the increase in $[Ca^{2+}]_i$. In LNCaP cells, both E_2 and DHT induce only calcium influx.

The nature of the Ca^{2+} channels activated by E_2 is not yet known. Electrophysiological experiments performed in our laboratory showed that LNCaP cells were devoid of the usual voltage-dependent Ca^{2+} channels. Thus, in LNCaP, as in other non-excitable cells, Ca^{2+} enters the cells through calcium channels that are neither voltage-dependent nor sensitive to dihydropyridine, as the application of PN200-110 did not affect the E_2-induced $[Ca^{2+}]_i$ rise (data not shown). We cannot exclude the possibility of other processes, such as the inhibition of a Ca^{2+} pump, inducing an accumulation of Ca^{2+} ions inside the cell. Steroids are a well-known stimulant to cell proliferation. Indeed, growth of LNCaP cancer cells is stimulated by estrogens (13, 10). In parallel experiments we confirmed a dose-dependent effect of E_2 on the proliferation of LNCaP cells, with a maximal increase at a concentration of 10 nmol/l E_2 (data not shown). Conversely, a higher dose of E_2 (10 μmol/l) has been reported to inhibit the growth of androgen-insensitive cells. This is possibly due to an effect on the cell membrane (37), because these cells are

devoid of classical estrogen nuclear receptors (38). Furthermore, estrogens are able to induce apoptosis in vivo in an androgen-sensitive rat prostatic adenocarcinoma (39). Thus, effects of estrogen on cell proliferation and death appear to be dissociated, and further study is obviously required to relate the increase in calcium influx induced by estrogen to the phenotypic effects of estrogen treatments.

In conclusion, the data presented in this study show a rapid membrane effect of E_2 in LNCaP cells, which suggests that the action of E_2 occurs, at least in part, independently of nuclear androgen and estrogen receptors and thus through a non-genomic site that remains to be characterized. Several hypotheses may explain how this receptor is related to calcium entry and, in particular, the possibility that this receptor may be a Ca^{2+} channel or a binding site on a Ca^{2+} channel, as described for other ions. These hypotheses are currently under investigation in our laboratory.

Acknowledgments. We would like to thank Professor J André (Unité Inserm 329, Lyon) for providing the LNCaP cell line, MF Odessa for cell cultures and D Varoqueaux for the illustrations. This research was supported in part by grants from Association pour la Recherche sur le Cancer (ARC) and Association pour la Recherche sur les Tumeurs de la Prostate (ARTP).

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Received January 22nd, 1996
Accepted April 29th, 1996