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Mechanism of Estrogens-Induced Increases in Intracellular Ca^{2+} in PC3 Human Prostate Cancer Cells

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BACKGROUND. The effect of estrogens (diethylstilbestrol [DES], 17β-estradiol) on intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) in hormone-insensitive PC3 human prostate cancer cells was examined.
METHODS. $[Ca^{2+}]_i$ changes in suspended cells were measured by using the Ca^{2+}-sensitive fluorescent dye fura-2.
RESULTS. Estrogens (1–20 μM) increased $[Ca^{2+}]_i$ concentration-dependently with DES being more potent. Ca^{2+} removal inhibited 50 ± 10% of the signal. In Ca^{2+}-free medium, pretreatment with 20 μM estrogens abolished the $[Ca^{2+}]_i$ increases induced by 2 μM carbonylcyanide *m*-chlorophenylhydrazone (CCCP, a mitochondrial uncoupler) and 1 μM thapsigargin (an endoplasmic reticulum Ca^{2+} pump inhibitor), but pretreatment with CCCP and thapsigargin did not alter DES-induced Ca^{2+} release and partly inhibited 17β-estradiol-induced Ca^{2+} release. Addition of 3 mM Ca^{2+} increased $[Ca^{2+}]_i$ in cells pretreated with 1–20 μM estrogens in Ca^{2+}-free medium. Pretreatment with 1 μM U73122 to block phospholipase C-coupled inositol 1,4,5-trisphosphate formation did not alter estrogens-induced Ca^{2+} release. The effect of 20 μM estrogen on $[Ca^{2+}]_i$ was not affected by pretreatment with 0.1 μM estrogens.
CONCLUSIONS. Estrogen induced significant Ca^{2+} release and Ca^{2+} influx in an inositol 1,4,5-trisphosphate-independent manner in PC3 cells. These effects of estrogens on Ca^{2+} signaling appear to be nongenomic. *Prostate 47:141–148, 2001.* © 2001 Wiley-Liss, Inc.

KEY WORDS: **17β-estradiol; Ca^{2+}; diethylstilbestrol; PC3; prostate cancer cells**

INTRODUCTION

Carcinoma of the prostate was one of the first forms of cancer shown to be responsive to hormonal manipulation. Because androgens are required for normal growth of prostate cells, low-dose estrogen therapy has resulted in prolonged improvement in patients with bone lesions [1]. However, many serious dose-dependent side effects may occur during estrogen therapy such as postmenopausal bleeding, nausea, breast tenderness, hyperpigmentation, migraine headaches, cholestasis, hypertension, and gallbladder disease. The cause of these side effects is unclear at all, and may involve cytotoxicity or alterations in cell function in a manner independent of action on estrogen receptors. Currently, the two major drugs used in estrogen therapy are diethylstilbestrol (DES) and 17β-estradiol.

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An increase in cytosolic free Ca^{2+} concentrations ($[Ca^{2+}]_i$) is a pivotal signal for regulation of function and growth in all cells [2–4]. In hormone-insensitive PC3 human prostate cancer cells, $[Ca^{2+}]_i$ has been shown to be increased by various endogenous and exogenous agents such as ATP/UTP, bombesin, calcitonin, endothelin, gastrin-releasing peptide, histamine, lysophosphatidic acid, thapsigargin and analogs, and thrombin [5–8]. However, the mechanism underlying the $[Ca^{2+}]_i$ increase in PC3 cells is not clear. Apoptosis has become a new target for cancer therapy [9], and abnormal Ca^{2+} signaling is a central feature of tumor cells and a potential target for cancer therapy [10]. Evidence shows that prolonged increases in $[Ca^{2+}]_i$ lead to apoptosis [11,12]. In PC3 cells, thapsigargin and analogs (inhibit the endoplasmic reticulum Ca^{2+} pump leading to $[Ca^{2+}]_i$ increases) were found to cause apoptosis [6,7,13], several apoptosis inducers were shown to activate Ca^{2+}-permeable cation channels [14], and Ca^{2+} influx blockers were found to suppress the proliferative and metastatic potential of PC3 cells [10].

While it was shown that PC3 cells possess genomic estrogen receptors [15], the effect of DES and 17β-estradiol on Ca^{2+} homeostasis was unknown in this cell type. DES (10 μM) was found to alter growth cone morphology, with collapse of growth cone filopodia and neurite retraction [16], and to induce renal cell carcinoma in hamster [17–19]. In coronary smooth muscle 17β-estradiol was shown to suppress $[Ca^{2+}]_i$ increases at a concentration of 0.6 μM [20] and to alter K^+ channels at a concentration of 30 μM [21,22]. 17β-estradiol and other neuroactive steroids like testosterone, pregnenolone sulfate, and dehydroepiandrosterone sulfate were found to regulate the activity of plasma membrane Ca^{2+}-ATPase [23]. Further, 17β-estradiol causes nongenomic inhibition of human P2X7 purinoceptors [24].

The present study is the first to investigate the effect of DES and 17β-estradiol on $[Ca^{2+}]_i$ in PC3 human prostate cancer cells. By using fura-2 as a Ca^{2+} probe, it was found that both estrogens caused significant increases in $[Ca^{2+}]_i$. The concentration-response relationship has been established, and the sources and the mechanisms of the $[Ca^{2+}]_i$ increase have been explored.

MATERIALS AND METHODS

Cell Culture

PC3 human prostate cancer cells were cultured in 93% Ham's F12 medium plus 7% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were kept at 37°C in 5% CO_2 containing humidified air.

Solutions

Ca^{2+} medium (pH 7.4) contained (in mM): NaCl 140; KCl 5; $MgCl_2$ 1; $CaCl_2$ 1.8; HEPES 10; glucose 5. Ca^{2+}-free medium contained no added Ca^{2+} plus 1 mM EGTA to chelate residual Ca^{2+}. The experimental solution contained less than 0.1% of solvent (dimethyl sulfoxide or ethanol) which did not affect $[Ca^{2+}]_i$ ($n = 3$).

Optical Measurements of $[Ca^{2+}]_i$

Trypsinized cells (10^6/ml) were loaded with the ester form of fura-2, fura-2/AM (2 μM) for 30 min at 25°C in Ca^{2+} medium. Cells were washed and resuspended in Ca^{2+} medium before use. Fura-2 fluorescence measurements were performed in a water-jacketed cuvette (25°C) with continuous stirring; the cuvette contained 1 ml of medium and 0.5 million cells. Fluorescence was monitored with a Shimadzu RF1503PC spectrofluorophotometer (Shimadzu Corporation, Kyoto, Japan) by recording excitation signals at 340 and 380 nm and emission signal at 510 nm at 1 sec intervals. Maximum and minimum fluorescence values were obtained by adding 0.1% Triton X-100 and 20 mM EGTA sequentially at the end of each experiment. $[Ca^{2+}]_i$ was calculated as described previously assuming a K_d of 155 nM [25].

Chemical Reagents

The reagents for cell culture were from Gibco (Gaithersburg, MD). Fura-2/AM was from Molecular Probes (Eugene, OR). Estrogens and androgens were from Sigma-Aldrich Co. (St. Louis, MO). U73122 (1-(6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino) hexyl)-1H-pyrrole-2,5-dione) was from Biomol (Plymouth Meeting, PA). The other reagents were from Sigma.

Statistical Analyses

Data were reported as the means ± SEM ($n = 4$–6). Statistical comparisons were determined by using the Student t test or ANOVA tests and significance was accepted when $P < 0.05$.

RESULTS

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

In normal Ca^{2+} medium, DES (1–20 μM) increased $[Ca^{2+}]_i$ concentration-dependently (Fig. 1A; traces a–c). The basal $[Ca^{2+}]_i$ was 85 ± 3 nM ($n = 6$). The response induced by 40 μM DES was similar to that induced by 20 μM DES (trace a). At a concentration of 0.1 μM DES had no effect (trace d). At a concentration of 20 μM, DES induced a $[Ca^{2+}]_i$ increase which

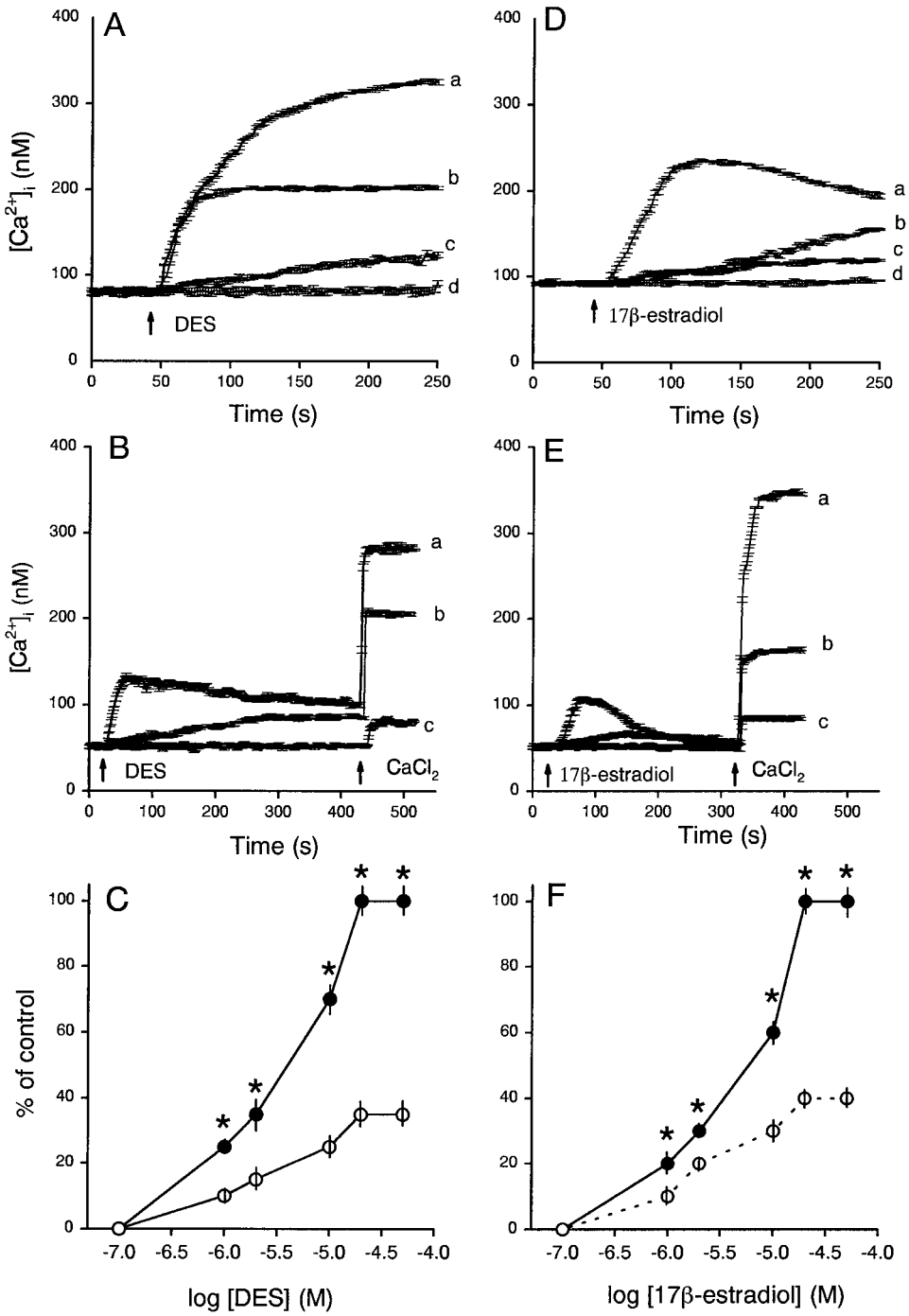

Fig. 1. Effects of estrogens on $[Ca^{2+}]_i$ in fura-2-loaded PC3 cells. (**A**) DES-induced $[Ca^{2+}]_i$ increases in Ca^{2+} medium. Concentrations of DES were 20 (trace a), 10 (trace b), 1 (trace c), and 0.1 (trace d) μM, respectively. (**B**) Effect of extracellular Ca^{2+} removal on DES-induced $[Ca^{2+}]_i$ increases and the effect of reintroduction of Ca^{2+}. DES (10–20 μM) was added at 30 sec in Ca^{2+}-free medium followed by addition of 3 mM $CaCl_2$ at 440 sec. The concentration of DES was 20, 10, and 0 μM in trace a, b, and c, respectively. (**C**) Concentration-response plots of DES-induced $[Ca^{2+}]_i$ increases in the presence (filled circles) and absence (open circles) of extracellular Ca^{2+}. The *y* axis is the percentage of control. Control is the net maximum $[Ca^{2+}]_i$ induced by 20 μM DES in Ca^{2+} medium. Data were mean ± SEM of 4–6 replicates. $^*P < 0.05$. (**D**–**F**) similar to (A–C) respectively, except that DES was substituted with 17β-estradiol.

reached a net (baseline subtracted) maximum value of 230 ± 5 nM ($n = 6$; $P < 0.05$), at the time point of 250 sec. Figure 1D shows the effect of 17β-estradiol on $[Ca^{2+}]_i$. Similar to DES, 17β-estradiol increased $[Ca^{2+}]_i$ concentration-dependently at a concentration range of 1–20 μM (traces a–c). At a concentration of 20 μM (trace a), 17β-estradiol induced a $[Ca^{2+}]_i$ increase with a net maximum value of 155 ± 4 nM ($n = 6$) at the time point of 105 ± 2 sec, followed by a slow decay which reached a net $[Ca^{2+}]_i$ of 101 ± 3 nM at the time point of 250 sec. Lower concentrations of 17β-estradiol (1–10 μM) induced a slow increase in $[Ca^{2+}]_i$. Diethylstilbestrol dipropionate (an inactive DES analog; 100 μM), testrosterone (1 nM), and the weak androgen: 4-androstene-3,17-dione (50 μM) had no effect on basal $[Ca^{2+}]_i$ ($n = 4$; not shown).

Effect of Extracellular Ca^{2+} Removal on the Estrogen Responses

Extracellular Ca^{2+} removal partly inhibited the DES-induced $[Ca^{2+}]_i$ increase (Fig. 1B; time points between 30–430 sec). The basal $[Ca^{2+}]_i$ was 51 ± 2 nM ($n = 6$). The concentration-response relationships of DES-induced $[Ca^{2+}]_i$ increases in the presence (filled circles) and absence (open circles) of Ca^{2+} are shown in Figure 1C. Ca^{2+} removal reduced 1–40 μM DES-induced $[Ca^{2+}]_i$ increases by $65 \pm 6\%$ ($n = 5$; $P < 0.05$). Figure 1E shows that in Ca^{2+}-free medium, 17β-estradiol (1–20 μM) increased $[Ca^{2+}]_i$ concentration-dependently between time points of 30–330 sec. The $[Ca^{2+}]_i$ increase induced by 20 μM 17β-estradiol comprised a slow rise which reached a net maximum of 51 ± 1 nM ($n = 6$; $P < 0.05$). The responses induced by lower concentrations of 17β-estradiol consisted of a slow increase. The concentration response relationships of 17β-estradiol-induced $[Ca^{2+}]_i$ increases in the presence (filled circles) and absence (open circles) of Ca^{2+} are shown in Figure 1F. Ca^{2+} removal reduced 1–40 μM 17β-estradiol-induced $[Ca^{2+}]_i$ increases by $55 \pm 5\%$ ($n = 4$; $P < 0.05$).

Effect of Estrogens on Ca^{2+} Influx

Depletion of intracellular Ca^{2+} stores often leads to Ca^{2+} influx via capacitative Ca^{2+} entry in many cell types [26]. Capacitative Ca^{2+} entry is usually measured by researchers by reintroduction of Ca^{2+} following depleting Ca^{2+} stores with the tested agent in Ca^{2+}-free medium. Figure 1B shows that in Ca^{2+}-free medium, after pretreatment with 1–20 μM DES for 400 sec, addition of 3 mM $CaCl_2$ increased $[Ca^{2+}]_i$ concentration-dependently. Three mM Ca^{2+} was added to Ca^{2+}-free medium which contained 1 mM EGTA to achieve a free Ca^{2+} concentration in the medium of around 2 mM. The Ca^{2+}-induced Ca^{2+} entry after pretreatment with 20 μM DES had a net maximum value of 241 ± 12 nM (trace a) which was 10-fold higher than control (trace c; adding Ca^{2+} alone without DES pretreatment; 24 ± 2 nM; $n = 5$; $P < 0.05$). Similarly, after 17β-estradiol pretreatment for 300 sec, addition of 3 mM $CaCl_2$ increased $[Ca^{2+}]_i$ concentration-dependently between 1–20 μM (Fig. 1E). The Ca^{2+}-induced Ca^{2+} entry induced by 20 μM 17β-estradiol had a net maximum value of 299 ± 3 nM (trace a) which was 12-fold higher than control (trace c; 23 ± 2 nM; $n = 5$; $P < 0.05$).

Intracellular Ca^{2+} Stores Involved in Estrogen Responses

The contribution of the Ca^{2+} stores in mitochondria and the endoplasmic reticulum was examined. Carbonylcyanide *m*-chlorophenylhydrazone (CCCP) is a mitochondrial uncoupler which was shown to release mitochondrial Ca^{2+} in renal cells [27]. Thapsigargin inhibits the endoplasmic reticulum Ca^{2+} pump leading to passive leakage of stored Ca^{2+} and, in turn, a $[Ca^{2+}]_i$ increase [28]. Figure 2A shows that in Ca^{2+}-free medium, addition of 2 μM CCCP induced an immediate increase in $[Ca^{2+}]_i$ with a net peak value of 36 ± 2 nM ($n = 6$; $P < 0.05$). The Ca^{2+} signal gradually decayed and nearly returned to baseline within 150 sec. Subsequently added 1 μM thapsigargin induced a $[Ca^{2+}]_i$ increase with a net peak value of 61 ± 2 nM, and the Ca^{2+} signal decayed slowly and reached a plateau within 200 sec. This suggests that by the time point of 700 sec a large portion of the Ca^{2+} stored in the mitochondria and the endoplasmic reticulum had been depleted. However, 20 μM DES added at 730 sec induced a $[Ca^{2+}]_i$ increase with a net maximum value of 41 ± 2 nM which was $57 \pm 2\%$ of the control response (Fig. 2B; 71 ± 3 nM; $n = 6$; $P < 0.05$). Figure 2B shows that 1 μM thapsigargin or 2 μM CCCP failed to increase $[Ca^{2+}]_i$ after pretreatment with 20 μM DES for 300 sec ($n = 5$).

The same strategy was applied to study the stores for 17β-estradiol-induced Ca^{2+} release. Figure 2C shows that after pretreatment with 2 μM CCCP and 1 μM thapsigargin for 700 sec, 20 μM 17β-estradiol induced a tiny $[Ca^{2+}]_i$ increase with a net peak value of 12 ± 2 nM which was $17 \pm 3\%$ of the control response (71 ± 5 nM; Fig. 2D; $n = 4$; $P < 0.05$). In contrast, Figure 2D shows that pretreatment with 20 μM 17β-estradiol for 400 sec prevented 1 μM thapsigargin or 2 μM CCCP from increasing $[Ca^{2+}]_i$.

Mechanisms of Estrogens-Induced Ca^{2+} Release

Experiments were performed to examine whether estrogens release Ca^{2+} via stimulating inositol 1,4,

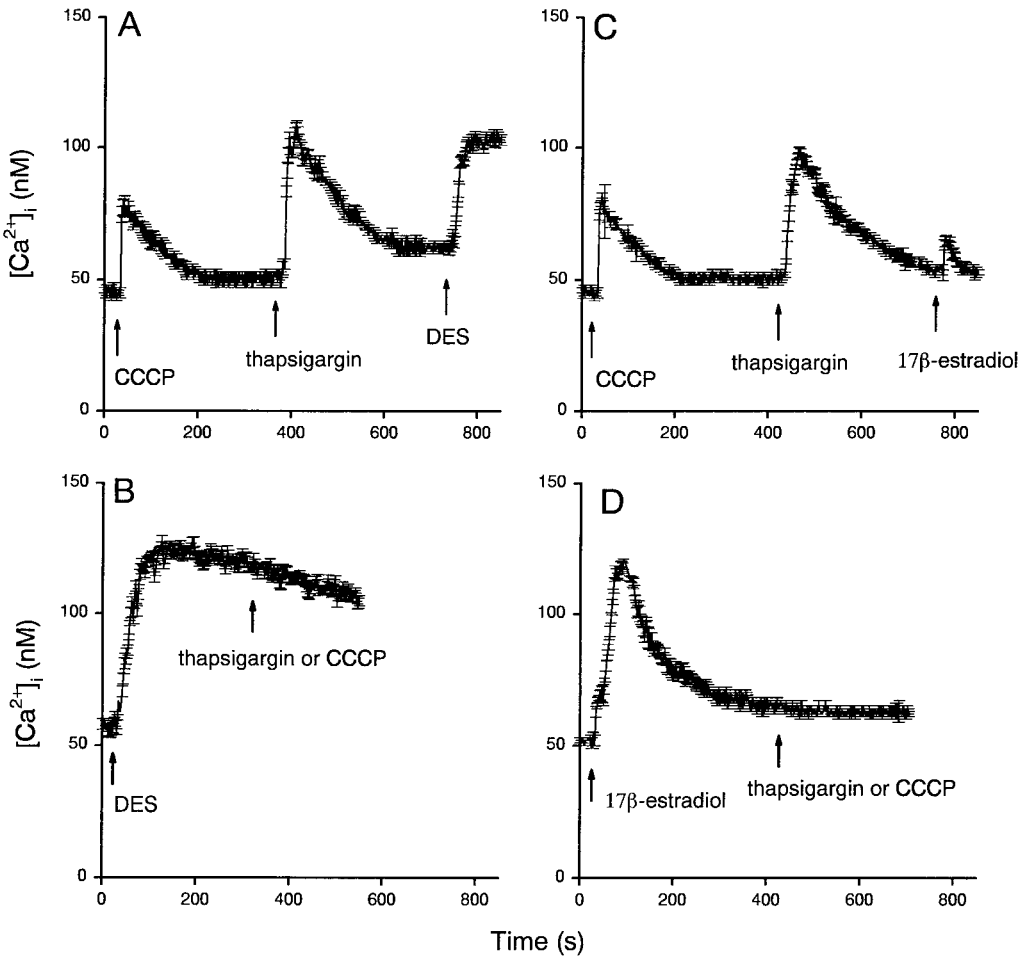

Fig. 2. Intracellular Ca^{2+} stores of estrogens-induced $[Ca^{2+}]_i$ increases. The experiments were performed in Ca^{2+}-free medium. Drugs were added at the time points indicated by arrows. The concentration of drugs was CCCP, 2 μM; thapsigargin, 1 μM; DES and 17β-estradiol, 20 μM. Data were mean ± SEM of 4–6 replicates.

5-trisphosphate formation, by exploring the effect of inhibiting phospholipase C on estrogens-induced $[Ca^{2+}]_i$ increases. Figure 3A shows that in Ca^{2+}-free medium 10 μM ATP, a well-established phospholipase C-coupled Ca^{2+} mobilizer, induced a transient $[Ca^{2+}]_i$ rise with a net maximum value of 45 ± 2 nM (n = 5). This suggests that PC3 cells possess phospholipase C-coupled Ca^{2+} mobilizing machinery. Figure 3B shows that pretreatment with 1 μM U73122, a phospholipase C inhibitor [29], for 190 sec abolished 10 μM ATP-induced $[Ca^{2+}]_i$ increases (n = 5). U73343, an inactive U73122 analog, did not alter ATP-induced $[Ca^{2+}]_i$ increases (n = 5; not shown). This suggests that phospholipase C was inhibited by U73122. DES (20 μM) added at 320 sec induced a $[Ca^{2+}]_i$ increase indistinguishable from control shown in Figure 1B (trace a; n = 4). Similarly, U73122 did not affect 20 μM 17β-estradiol-induced Ca^{2+} release (n = 6; not shown).

The possibility that estrogens induce $[Ca^{2+}]_i$ increases in a receptor-dependent manner was examined. Figure 3C shows that in Ca^{2+}-free medium, pretreatment with 0.1 μM DES for about 3 min to desentisize DES-sensitive receptors did not alter the $[Ca^{2+}]_i$ increase induced by subsequently added 20 μM DES (n = 4; $P > 0.05$). Similar results were found for 17β-estradiol.

DISCUSSION

This study is the first to examine the effect of estrogens on PC3 human prostate cancer cells. The results suggest that DES and 17β-estradiol caused a concentration-dependent $[Ca^{2+}]_i$ increase between 1–20 μM. It was shown previously [30] that in patients of prostatic cancer receiving DES, the maximum plasma concentration of DES and its glucuronide conjugate fluctuate between 1 and 32 μg/ml (equivalent to about 4–120 μM). Thus, our results suggest that the clinical plasma level of DES may alter Ca^{2+} signaling in patient's prostatic cancer cells. The data in Figure 3C

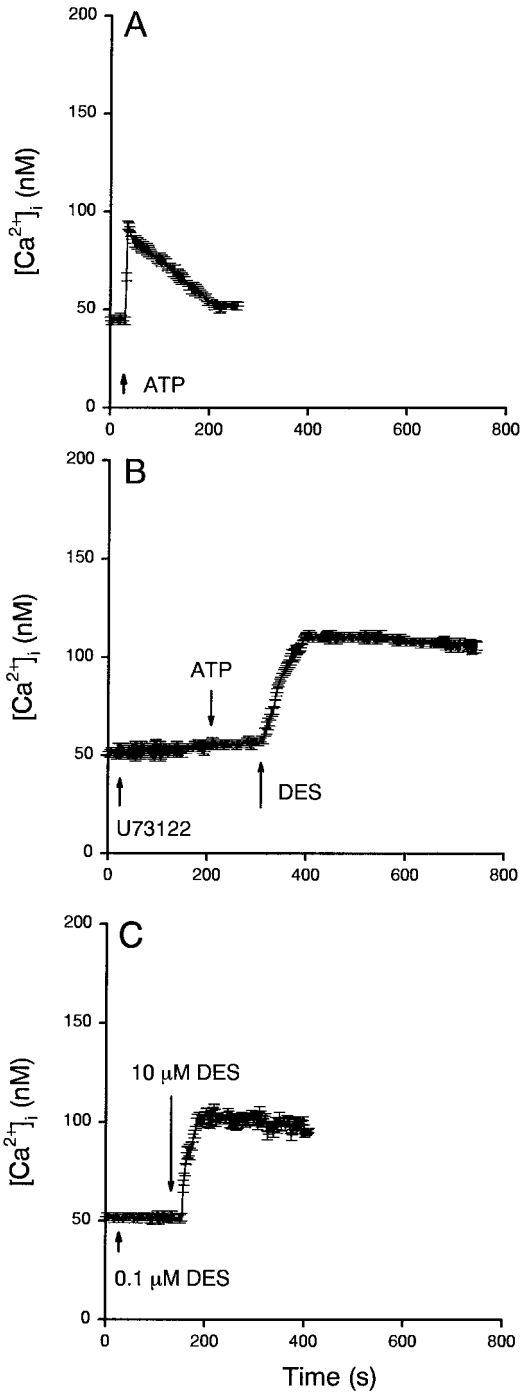

Fig. 3. The mechanism of estrogens-induced Ca^{2+} release. The experiments were performed in Ca^{2+}-free medium. (**A**) 10 μM ATP was added at 30 sec. (**B**) 2 μM U73122, 10 μM ATP, and 20 μM DES were added at the time points indicated by arrows. (**C**) 0.1 μM DES was added at 30 sec followed by 10 μM DES at 180 sec. Data were mean ± SEM of 4–6 replicates.

suggest that DES induces $[Ca^{2+}]_i$ increases in a receptor-independent (nongenomic) manner. This is consistent with the rapidity of the estrogens-induced $[Ca^{2+}]_i$ increase. Previous evidence also shows that 17β-estradiol increases $[Ca^{2+}]_i$ in chicken granulosa cells in a nongenomic manner [31]. Our data shows that in Ca^{2+} medium, the $[Ca^{2+}]_i$ increases induced by estrogens were prolonged and did not significantly decay during the measurement. This suggests that estrogens may have significant impact on cell function by altering Ca^{2+} signaling and activating Ca^{2+}-dependent enzymes. Because prolonged elevations in $[Ca^{2+}]_i$ are closely linked to cell dysfunction and death [9,10], the effect of estrogens on $[Ca^{2+}]_i$ may explain their cytotoxicity.

Estrogens-induced Ca^{2+} signals are approximately equally contributed by Ca^{2+} influx and release, because Ca^{2+} removal inhibited about half of the estrogen response in Ca^{2+} medium. It appears that DES-induced $[Ca^{2+}]_i$ increases comprise several pools including the ones in the mitochondria, the endoplasmic reticulum, and unknown stores. This is because in Ca^{2+}-free medium, pretreatment with CCCP and thapsigargin to deplete the Ca^{2+} stores in the mitochondria and the endoplasmic reticulum, respectively, did not alter DES-induced $[Ca^{2+}]_i$ increases, while pretreatment with DES abolished the $[Ca^{2+}]_i$ increases-induced by CCCP and thapsigargin. In contrast, 17β-estradiol released Ca^{2+} mainly from the mitochoindria and the endoplasmic reticulum because pretreatment with CCCP and thapsigargin nearly abolished 17β-estradiol-induced Ca^{2+} release. This is consistent with the data that DES induced a greater Ca^{2+} release than 17β-estradiol. Combined with the data in Figure 1, it is concluded that DES is more potent than 17β-estradiol in releasing Ca^{2+} and causing Ca^{2+} influx in PC3 cells.

It seems that estrogens release Ca^{2+} in a fashion independent of the phospholipase C/inositol 1,4,5-trisphosphate system, because estrogens released Ca^{2+} normally when phospholipase C activity was inhibited. The exact mechanism underlying estrogens-induced Ca^{2+} release from multiple stores is unclear, but it is likely that estrogens may act by inhibiting the Ca^{2+} pumps on the membranes of internal stores as it was shown previously that DES inhibited the Ca^{2+}-ATPase activity of sarcoplasmic reticulum membranes [32].

Another question was how estrogens induce Ca^{2+} influx. The results suggest that in Ca^{2+}-free medium, after estrogens had discharged Ca^{2+} stores for 5–6 min, addition of Ca^{2+} evoked a concentration-dependent increase in $[Ca^{2+}]_i$. This suggests that estrogens may induce Ca^{2+} influx via capacitative Ca^{2+} entry. However, the results do not exclude the possibility that estrogens may directly cause Ca^{2+} entry in a manner dissociated from Ca^{2+} store depletion.

Collectively, this study has explored the effect of DES and 17β-estradiol on $[Ca^{2+}]_i$ in hormone-insensi-

tive human prostate cancer cells, and has examined the underlying mechanisms. The results suggest that both estrogens induce considerable increases in $[Ca^{2+}]_i$ after immediate exposure. These estrogen receptor-independent actions of DES and 17β-estradiol may contribute to the diverse, severe side effects observed in patients taking a rather high dose of the drugs.

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