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Effect of clomiphene on Ca^{2+} movement in human prostate cancer cells

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Abstract

The effect of clomiphene, an ovulation-inducing agent, on cytosolic free Ca^{2+} levels ($[Ca^{2+}]_i$) in populations of PC3 human prostate cancer cells was explored by using fura-2 as a Ca^{2+} indicator. Clomiphene at concentrations between 10-50 μM increased $[Ca^{2+}]_i$ in a concentration-dependent manner. The $[Ca^{2+}]_i$ signal was biphasic with an initial rise and a slow decay. Ca^{2+} removal inhibited the Ca^{2+} signal by 41%. Adding 3 mM Ca^{2+} increased $[Ca^{2+}]_i$ in cells pretreated with clomiphene in Ca^{2+}-free medium, confirming that clomiphene induced Ca^{2+} entry. In Ca^{2+}-free medium, pretreatment with 50 μM brefeldin A (to permeabilize the Golgi complex), 1 μM thapsigargin (to inhibit the endoplasmic reticulum Ca^{2+} pump), and 2 μM carbonylcyanide m-chlorophenylhydrazone (to uncouple mitochondria) inhibited 25% of 50 μM clomiphene-induced store Ca^{2+} release. Conversely, pretreatment with 50 μM clomiphene in Ca^{2+}-free medium abolished the $[Ca^{2+}]_i$ increase induced by brefeldin A, thapsigargin or carbonylcyanide m-chlorophenylhydrazone. The 50 μM clomiphene-induced Ca^{2+}release was unaltered by inhibiting phospholipase C with 2 μM 1-(6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1H-pyrrole-2,5-dione (U73122). Trypan blue exclusion assay suggested that incubation with clomiphene (50 μM) for 2-15 min induced time-dependent decrease in cell viability by 10-50%. Collectively, the results suggest that clomiphene induced $[Ca^{2+}]_i$ increases in PC3 cells by

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releasing store Ca^{2+} from multiple stores in an phospholipase C-independent manner, and by activating Ca^{2+} influx; and clomiphene was of mild cytotoxicity.

Keywords: $[Ca^{2+}]_i$; Clomiphene; Fura-2; PC3 cells; Prostate

Introduction

Estrogen receptor modulators are structurally diverse non-steroidal compounds that are thought to bind to estrogen receptors and produce estrogen agonist effects in some tissues and estrogen antagonist effects in others. These drugs include clomiphene, tamoxifen, toremifene, and raloxifene. They are clinically prescribed for treating many estrogen-related diseases, including post-menopausal osteoporosis, hormone-dependent cancers, and cardiovascular disease [1,2].

Clomiphene is mostly used for the induction of ovulation in sub-fertile women attempting pregnancy [3]. However, clomiphene also alters male reproductive function. In rats, clomiphene was shown to decrease the serum levels of gonadotropins coupled to reduced testosterone secretion [4]. In men, clomiphene administration resulted in an increase in serum levels of follicle-stimulating hormone (FSH), luteinizing hormone (LH), testosterone, estradiol, and sperm count. The spermatic fluid concentrations of zinc and magnesium ions were also increased [5].

Clomiphene was found to induce regressive histological changes in the prostate and ampullary gland accompanied by a decrease in the weight in the musk screw [6]. However, the effect of clomiphene on signal transduction and function of human prostate cells has not been explored previously.

We have recently shown that clomiphene induced an increase in $[Ca^{2+}]_i$ in osteoblasts and bladder cancer cells [7,8]. The present study was aimed to examine the effect of clomiphene on cytosolic free Ca^{2+} levels ($[Ca^{2+}]_i$) in human prostate cancer cells. Ca^{2+} ions play a key role as a second messenger in various cell processes [9]. Stimulation of plasma membrane receptors coupled to phospholipase C results in an increase in $[Ca^{2+}]_i$ in most cell types via store Ca^{2+} release and/or extracellular Ca^{2+} influx [10,11]. A regulated $[Ca^{2+}]_i$ increase is essential for normal cell function; however, unregulated or prolonged elevations in $[Ca^{2+}]_i$ may injure or even kill the cell [12]. PC3 human prostate cancer cells were chosen for this study because it has been shown that $[Ca^{2+}]_i$ in this cell line can be significantly increased by several compounds including estrogens such as 17β-estradiol and diethylstilbestrol [13,14].

The Ca^{2+}-sensitive fluorescent probe fura-2 was used to measure $[Ca^{2+}]_i$ changes in populations of PC3 cells. The results suggest that clomiphene induced significant increases in $[Ca^{2+}]_i$. The concentration-response relationship has been established, and the underlying mechanisms of the clomiphene response, such as Ca^{2+} sources and the role of phospholipase C have been evaluated.

Materials and methods

Cell culture

PC3 human prostate cancer cells were cultured in 93% Ham's F12 medium plus 7% fetal bovine serum at 37 °C in 5% CO_2-containing humidified air.

Solutions used in $[Ca^{2+}]_i$ measurements

Ca^{2+}-containing medium (pH 7.4) had 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM Hepes, and 5 mM glucose. In Ca^{2+}-free medium, Ca^{2+} was substituted with 1 mM EGTA. Drugs were dissolved in water, ethanol or dimethyl sulfoxide as stock solutions. The concentration of organic solvents in the $[Ca^{2+}]_i$ measurements did not exceed 0.1% and did not alter basal $[Ca^{2+}]_i$ (n = 4).

$[Ca^{2+}]_i$ measurements

Trypsinized cells (10^6/ml) were allowed to recover in serum-free culture medium for 1 hr before loading with 2 μM fura-2/AM for 30 min at 25 °C in the same medium. Cells were washed and resuspended in Ca^{2+}-containing medium. 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 RF-5301PC spectrofluorophotometer (Shimadzu Kyoto, Japan) by continuously recording excitation signals at 340 nm and 380 nm and emission signal at 510 nm at 1-s intervals. Maximum and minimum fluorescence values were obtained by adding 0.1% Triton X-100 (plus 5 mM $CaCl_2$) and 10 mM EGTA sequentially at the end of each experiment. $[Ca^{2+}]_i$ was calculated as described previously assuming a K_d of 155 nM [15–17].

Viability assay

Fifty μl of cell suspension was mixed with 50 μl trypan blue isotonic solution (0.2%; w/v) in the presence or absence of 50 μM clomiphene; and cell viability was determined on a hemocytometer under a microscope.

Chemicals

All chemicals used in culture were obtained from Gibco (Grand Island, NY). The other drugs were obtained from Sigma (St. Louis, MO).

Statistics

The data were mean ± SEM of 4–6 replicates. Statistical analysis was made by Student's *t*-test. $P < 0.05$ was considered significant.

Results

Effect of clomiphene on $[Ca^{2+}]_i$ in PC3 cells

Fig. 1A shows that in Ca^{2+}-containing medium, clomiphene at concentrations of 50 μM (trace a), 20 μM (trace b), and 10 μM (trace c) induced a significant increase in basal $[Ca^{2+}]_i$ in a concentration-dependent manner. At a concentration of 1 μM, clomiphene had no effect (d). The $[Ca^{2+}]_i$ signal saturated at 50 μM of clomiphene because 100 μM clomiphene did not induce a greater response. The basal $[Ca^{2+}]_i$ was 51 ± 2 nM (n = 6). Over a time period of 250 s, the $[Ca^{2+}]_i$ signals induced by 20–50 μM clomiphene comprised an initial rise, a slow decay and a sustained phase. The Ca^{2+} signal induced by 10 μM clomiphene was composed of a gradual increase and a sustained phase. At a concentration of 50 μM, clomiphene induced a $[Ca^{2+}]_i$ increase that reached a net (baseline subtracted) maximum value of 132 ± 3 nM after a time lapse of 25 ± 2 s (n = 5). The $[Ca^{2+}]_i$ signal gradually decayed to an elevated phase with a net value of 58 ± 3 nM at the time point of 250 s. Fig. 1C shows the concentration-response curve of the clomiphene-induced $[Ca^{2+}]_i$ increases (filled circles). The curve suggests an EC_{50} value of about 30 μM.

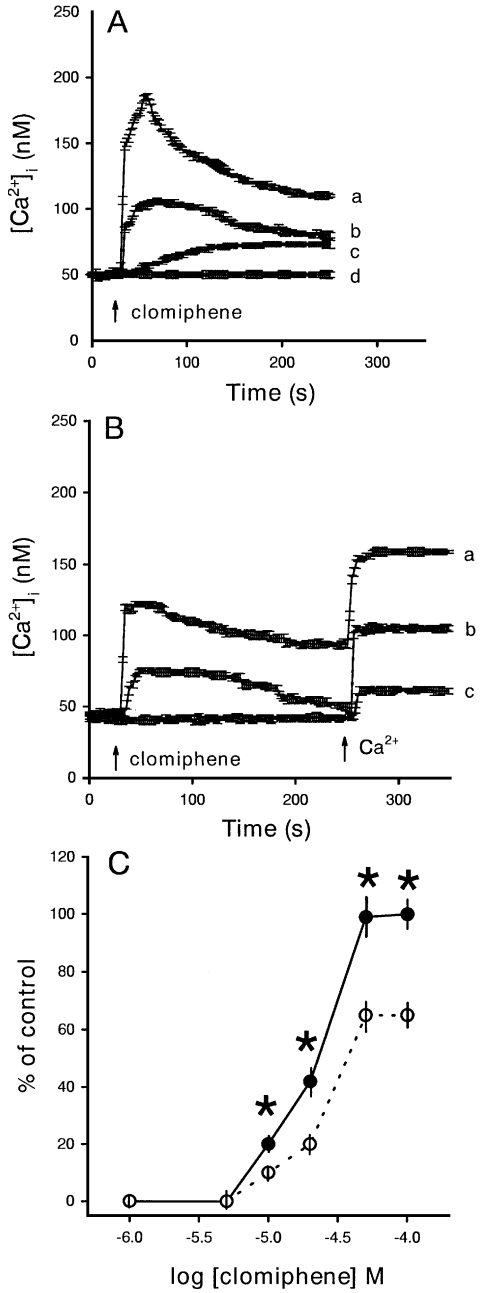
Effect of removing extracellular Ca^{2+} on clomiphene-induced $[Ca^{2+}]_i$ increases

This set of experiments was performed to explore the relative contribution of extracellular Ca^{2+} influx and store Ca^{2+} release in clomiphene-induced $[Ca^{2+}]_i$ increases. Fig. 1B (time points between 10-250 s) shows that substituting extracellular Ca^{2+} with 1 mM EGTA significantly reduced clomiphene-induced $[Ca^{2+}]_i$ increases. The Ca^{2+} signal induced by 20 μM clomiphene (trace b) was composed of an initial rise that reached a net maximum value of 36 ± 3 nM (n = 6), and a slow decay to the pre-stimulatory baseline after the time point of 250 s. Fig. 1C shows the concentration-response curve of the clomiphene-induced $[Ca^{2+}]_i$ increases in Ca^{2+}-free medium (open circles). The data suggest that removing Ca^{2+} reduced 10-100 μM clomiphene-induced $[Ca^{2+}]_i$ increases by 41±2% (n = 5; $P < 0.05$).

Fig. 1B (time points between 250-350 s) further shows that after incubation with 20-50 μM clomiphene for several min in Ca^{2+}-free medium, addition of 3 mM Ca^{2+} induced an immediate increase in $[Ca^{2+}]_i$ with a net maximum of 62 ± 3 nM which was 3-fold higher

Fig. 1. Effect of clomiphene on $[Ca^{2+}]_i$ in PC3 cells. (A) Clomiphene-induced $[Ca^{2+}]_i$ increases in Ca^{2+}-containing medium. The concentration of clomiphene was 50 (trace a), 20 (trace b), and 10 (trace c) μM. The drug was added at 20 s. Trace d: control without clomiphene addition. At a concentration of 1 μM, clomiphene had no effect. (B) Effect of removing extracellular Ca^{2+} on clomiphene-induced $[Ca^{2+}]_i$ increases and the effect of adding back Ca^{2+}. The experiments were performed in Ca^{2+}-free medium. The concentration of clomiphene was 50 (trace a), 20 (trace b), and 0 (trace c) μM. Clomiphene was added at 20 s followed by 3 mM Ca^{2+} added at 250 s. (C) The concentration-response plots of clomiphene-induced Ca^{2+} signals in Ca^{2+}-containing medium (solid circles) and Ca^{2+}-free medium (open circles). Y axis was the percentage of control. Control was the net (baseline subtracted) maximum value of 50 μM clomiphene-induced $[Ca^{2+}]_i$ increases in Ca^{2+}-containing medium. Data were mean ± SEM of 4–6 replicates. *$P < 0.05$ between filled circles and open circles.

than control (21 ± 2 nM; n = 5; $P < 0.05$). This confirms that clomiphene induced Ca^{2+} influx. This Ca^{2+}influx was not sensitive to Ca^{2+} entry blockers such as nifedipine, verapamil and diltiazem (all at 10 μM) added 30 s prior to Ca^{2+} (n = 4; not shown).



Intracellular Ca^{2+} stores of clomiphene-induced $[Ca^{2+}]_i$ increases

The source of store Ca^{2+} for the clomiphene-induced $[Ca^{2+}]_i$ increase was explored. Thapsigargin, carbonylcyanide m-chlorophenylhydrazone, and brefeldin A were used to deplete store Ca^{2+} in the endoplasmic reticulum, mitochondria, and the Golgi complex, respectively. Thapsigargin is an inhibitor of the endoplasmic reticulum Ca^{2+} pump [18], and can increase $[Ca^{2+}]_i$ in many cell types [7,8,13,14,16,17]. Carbonylcyanide m-hlorophenylhydrazone can release mitochondrial Ca^{2+} by uncoupling electron transport and oxidative phosphorylation [16,17]. Brefeldin A has been shown to permeabilize the Golgi complex membrane and release store Ca^{2+} [7,8]. Thapsigargin and carbonylcyanide m-chlorophenylhydrazone have been previously shown to release store Ca^{2+} in PC3 cells [13,14]. Fig. 2A shows that in Ca^{2+}-free medium, 50 μM clomiphene induced a Ca^{2+} signal with a net maximum value of 75 ± 3 nM (n = 5). After clomiphehe pretreatment for 280 s, thapsigargin (1 μM), carbonylcyanide m-chlorophenylhydrazone (2 μM), and brefeldin A (50 μM) failed to induce a $[Ca^{2+}]_i$ increase (n = 5). In contrast, Fig. 2B shows that 1 μM thapsigargin induced a $[Ca^{2+}]_i$ increase with a net maximum value of 129 ± 3 nM (n = 6). The Ca^{2+} signal was followed by a gradual decay and a sustained phase. After thapsigargin incubation for 360 s, addition of 50 μM clomiphene induced a $[Ca^{2+}]_i$ increase with a net maximum value of 55 ± 4 nM, which was 73 ± 2% ($P < 0.05$) of the control clomiphene response shown in Fig. 2A. This suggests that clomiphene released store Ca^{2+} from the endoplasmic reticulum and other pools. Fig. 2C shows that addition of brefeldin A (50 μM) induced an immediate increase in $[Ca^{2+}]_i$ that reached a net level of 31 ± 2 nM (n = 5) within 40 s. Subsequently added carbonylcyanide m-chlorophenylhydrazone (2 μM) released more Ca^{2+} and induced a further increase in $[Ca^{2+}]_i$ with a net value of 31 ± 2 nM (n = 5). Thapsigargin (1 μM) was subsequently added and induced a $[Ca^{2+}]_i$ increase with a net value of 61 ± 2 nM (n = 5). After depleting Ca^{2+} in the Golgi complex, mitochondria and the endoplasmic reticulum stores for several min, addition of 50 μM clomiphene induced a $[Ca^{2+}]_i$ increase indistinguishable from clomiphene-induced responses in Fig. 2B (n = 5; $P > 0.05$).

Effects of Ca^{2+} channel blockers on clomiphene-induced $[Ca^{2+}]_i$ increases

In Ca^{2+}-containing medium, the 50 μM clomiphene-induced $[Ca^{2+}]_i$ increase was unaffected by pretreatment with 1 μM of nifedipine, nimodipine, nicardipine, verapamil and diltiazem (n = 4; $P > 0.05$; not shown).

Effect of inhibiting phospholipase C on clomiphene-induced $[Ca^{2+}]_i$ increases

U73122 is an effective inhibitor of phospholipase C-dependent formation of inositol 1,4,5-trisphosphate in many cells types [19] including PC3 cells [13,14]. Fig. 3A shows that in Ca^{2+}-free medium, addition of 10 μM ATP, a phospholipase C-dependent agonist, induced a $[Ca^{2+}]_i$ increase with a net maximum value of 46 ± 2 nM (n = 5), suggesting the presence of phospholipase C-dependent Ca^{2+}mobilizing machinery in PC3 cells. Fig. 3B shows that addition of 2 μM U73122 for 3 min abolished ATP-induced $[Ca^{2+}]_i$ increases. Conversely,

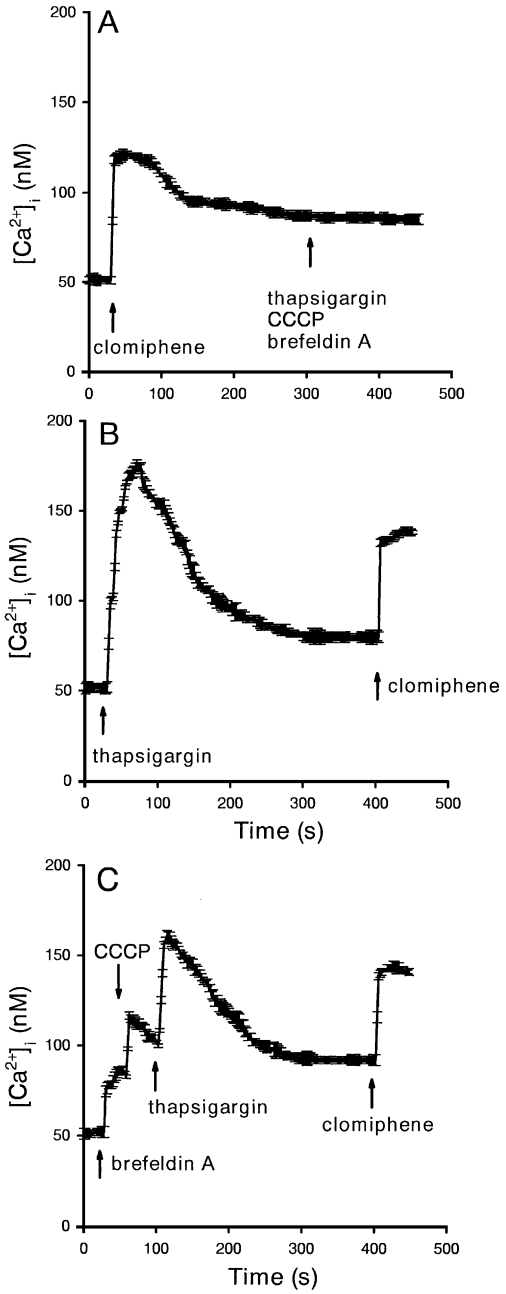


Fig. 2. Intracellular Ca^{2+} stores of clomiphene-induced $[Ca^{2+}]_i$ increases. The experiments were performed in Ca^{2+}-free medium. Clomiphene (50 μM), thapsigargin (1 μM), carbonylcyanide m-chlorophenylhydrazone (CCCP; 2 μM), brefeldin A (50 μM) were added at time points indicated by arrows. Data were mean ± SEM of 4–6 replicates.

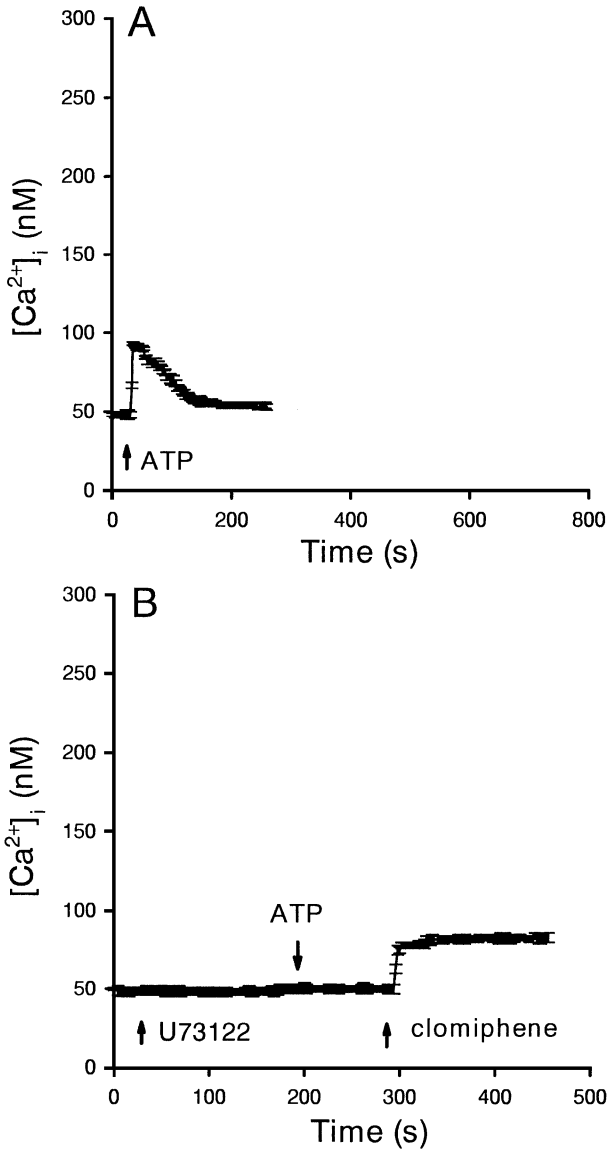


Fig. 3. Effect of inhibiting phospholipase C on clomiphene-induced Ca^{2+} release. Experiments were performed in Ca^{2+}-free medium. (A) ATP (10 μM) was added at 20 s. (B) U73122 (2 μM), ATP (10 μM), and clomiphene (20 μM) were added at time points indicated by arrows. Data were mean ± SEM of 4–6 replicates.

U73343, an inactive analogue of U73122 [19], did not alter ATP-induced $[Ca^{2+}]_i$ increases. This indicates that U73122 effectively suppressed phospholipase C activity. Fig. 3B shows that 20 μM clomiphene added after ATP induced a $[Ca^{2+}]_i$ increase similar to the control clomiphene response shown in Fig. 1B (trace b) ($n = 4$; $P > 0.05$).

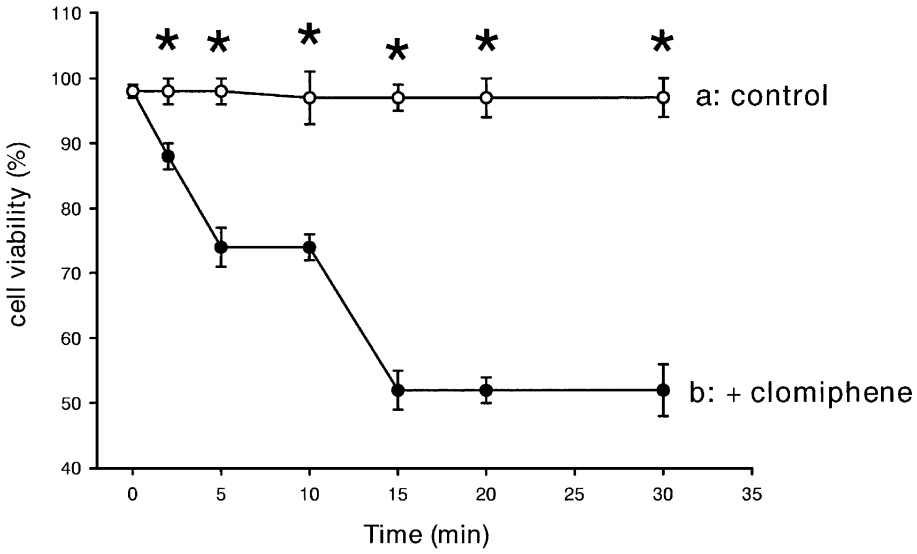


Fig. 4. Effect of clomiphene on cell viability. See Methods for trypan blue exclusion assay. Trace a (control): cell viability measured in the absence of clomiphene. Trace b: 50 μM clomiphene was added to cell suspension for 1, 2, 5, 10, 15, 20 or 30 min before trypan blue was added to determine cell viability. Data were mean ± SEM of 4–6 replicates. *$P < 0.05$ between open circles and filled circles at each time point.

Effect of acute exposure of clomiphene on cell viability

Trypan blue exclusion assay revealed that control cell viability was 98 ± 2% throughout the measurement of 30 min (Fig. 4, trace a). Incubation with 50 μM clomiphene for 2-15 min reduced cell viability by 10-50% ($n = 4$-6; $P < 0.05$) in a time-dependent manner. Increasing incubation time up to 30 min did not further reduce viability ($P > 0.05$).

Discussion

This study has explored the effect of clomiphene on $[Ca^{2+}]_i$ in human prostate cancer cells. Our data suggest that this drug, clinically used for ovulation induction, caused an immediate and significant increase in $[Ca^{2+}]_i$ in a concentration-dependent manner starting at a concentration of 10 μM, and the response saturated at 50 μM with an EC_{50} of about 30 μM. How clomiphene increased $[Ca^{2+}]_i$ is unclear. Clomiphene and a group of structurally similar non-steroidal, triphenolic compounds are known to affect some types of plasma membrane ion channels and other proteins through mechanisms that do not appear to involve their interactions with the estrogen receptor but could be the result of their effect on membrane lipid structure or fluidity [20].

The clomiphene-induced Ca^{2+} signal resulted from extracellular Ca^{2+} influx and store Ca^{2+} release because removing extracellular Ca^{2+} reduced 41% of the response. This reduction was not caused by store Ca^{2+} depletion as extracellular Ca^{2+} was substituted by 1 mM EGTA.

This is because addition of 3 mM Ca^{2+} after cells were bathed in Ca^{2+}-free medium for several min induced a significant $[Ca^{2+}]_i$ increase, suggesting Ca^{2+} influx really occurred during clomiphene stimulation. One characteristic of the clomiphene-induced Ca^{2+} entry is its insensitivity to voltage-gated Ca^{2+} entry blockers.

Our data suggest that clomiphene could completely deplete the Ca^{2+} stored in the endoplasmic reticulum, mitochondria and the Golgi complex, and could also release $[Ca^{2+}]_i$ from other unidentified compartments. This is because in Ca^{2+}-free medium, pretreatment with thapsigargin, carbonylcyanide m-chlorophenylhydrazone and brefeldin A to deplete Ca^{2+} from the endoplasmic reticulum, mitochondria and the Golgi complex, respectively, only partly inhibited the clomiphene-response; whereas pretreatment with clomiphene abolished the $[Ca^{2+}]_i$ increase induced by thapsigargin, carbonylcyanide m-chlorophenylhydrazone or brefeldin A. For comparison, the clomiphene-induced Ca^{2+} release was reduced by 85% and 51%, respectively, by depleting Ca^{2+} stored in endoplasmic reticulum, mitochondria and the Golgi complex, in human bladder cancer cells and osteoblasts [7,8]. These data suggest that multiple Ca^{2+} stores may contribute cooperatively to clomiphene-induced $[Ca^{2+}]_i$ increases in human cells from different tissues.

It appears that clomiphene did not employ inositol 1,4,5-trisphosphate as a second messenger to release store Ca^{2+} because the clomiphene-induced Ca^{2+} release was not affected when phospholipase C activity was suppressed. The mechanism underlying the action of clomiphene remains to be investigated. Evidence shows that clomiphene and other estrogenic compounds such as tamoxifen and toremifene can inhibit sarcoplasmic reticulum Ca^{2+} uptake in a concentration-dependent manner without inhibiting the ATPase activity of the sarcoplasmic reticulum Ca^{2+} pump [20].

Clomiphene and other estrogenic compounds (tamoxifen, 17β-estradiol, nafoxidine) have been shown to inhibit the growth of neuroblastoma cells by decreasing cell viability; and that both intracellular Ca^{2+} release and extracellular Ca^{2+} entry were thought to play a role in their growth regulation [21]. These data were consistent with our findings that clomiphene induced an acute decrease in the viability of PC3 prostate cells, accompanied by an increase in Ca^{2+} influx and store Ca^{2+} release. Indeed, in PC3 cells, thapsigargin and its analogues have been shown to induce cell dysfunction and apoptosis [22,23], and we have shown in this study and previous reports [13,14] that thapsigargin can induce significant $[Ca^{2+}]_i$ increases in PC3 cells. A potential clinical implication that may be useful for patients suffering from prostate carcinoma is that clomiphene may kill or inhibit prostate cancer cells via increasing $[Ca^{2+}]_i$.

Together, this study has explored the effect of clomiphene on $[Ca^{2+}]_i$ in human prostate cancer cells, and has evaluated the mechanisms. Due to the rapidity of the initiation of clomiphene-induced $[Ca^{2+}]_i$ increases (within seconds), it is unlikely that this action of clomiphene was via stimulation of conventional genomic estrogen receptors. Indeed, other estrogen-like compounds such as 17β-estradiol and diethylstilbestrol have been shown to increase $[Ca^{2+}]_i$ in PC3 cells via nongenomic pathways [14]; and that 17β-estradiol can influence oocyte cytoplasmic maturation via a nongenomic induction of $[Ca^{2+}]_i$ oscillations [24]. Because the concentration of clomiphene in patients taking large doses of the drug may reach μM ranges [25], and because that clomiphene was found to induce regressive histological changes and weight decrease in the prostate in an animal model [6], the possible

effect of clomiphene on $[Ca^{2+}]_i$ in prostate cells should be considered in evaluating the in vivo action of this drug when used at rather high dosage.

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