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Regulation of Intracellular Calcium in Human Breast Cancer Cells

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Regulation of intracellular Ca^{2+} in breast cancer may be important in modulating cell proliferation, differentiation, apoptosis, and cytotoxicity, as well as contributing to mechanisms of action of anticancer agents. One of these agents, the steroid hormone 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$], is intimately involved in maintaining cellular Ca^{2+} homeostasis. The purpose of this study was to investigate Ca^{2+} regulatory pathways in breast cancer cells and to determine the role of 1,25$(OH)_2D_3$ in modulating these pathways. We examined pathways for Ca^{2+} entry from the extracellular space and Ca^{2+} mobilization from intracellular stores in the estrogen-receptor negative human breast cancer cell line BT-20. Fluorescence digital video imaging and Ca^{2+} indicator fura-2 were employed to measure the concentration of intracellular free Ca^{2+} ($[Ca^{2+}]_i$) and Ca^{2+} responses at the single-cell level. We found that BT-20 breast cancer cells expressed nonselective, voltage-insensitive Ca^{2+} channels (VICC), as indicated by their permeability to Mn^{2+}, response to elevated extracellular Ca^{2+} with an increase in $[Ca^{2+}]_i$, blockage by La^{3+} and Ni^{2+}, and response to K^+ depolarization with a slight decrease in $[Ca^{2+}]_i$ and Ca^{2+} influx. There was no evidence for voltage- dependent Ca^{2+} channels in BT-20 cells. Endoplasmic reticulum Ca^{2+} stores comprised a major intracellular Ca^{2+} pool, as was evident after application of a Ca^{2+} ionophore ionomycin in nominally Ca^{2+}-free buffer to the cells with thapsigargin-depleted Ca^{2+} stores. Thapsigargin depletion of Ca^{2+} stores did not increase influx of extracellular Ca^{2+}, implying no significant activation of the capacitative Ca^{2+} entry. 1,25$(OH)_2D_3$ did not induce a rapid rise in $[Ca^{2+}]_i$, yet Ca^{2+} influx through VICC was increased. Treatment with 1,25$(OH)_2D_3$ for 4-72 h significantly increased the percentage of cells with a markedly elevated basal $[Ca^{2+}]_i$. Ca^{2+} response of those cells to thapsigargin was attenuated. Taken together, our findings show that VICC and the thapsigargin-sensitive endoplasmic reticulum Ca^{2+} stores are the principal pathways for Ca^{2+} entry and Ca^{2+} mobilization in the breast cancer cell line used in this study. 1,25$(OH)_2D_3$ rapidly increases Ca^{2+} influx through VICC and after a chronic treatment, depletes endoplasmic reticulum Ca^{2+} stores. Targeting of Ca^{2+} signaling mediated by VICC and endoplasmic reticulum Ca^{2+} stores may represent a novel approach to the treatment and chemoprevention of breast cancer.

Key Words: Intracellular Ca^{2+}; breast cancer cells; 1,25-dihydroxyvitamin D_3; Ca^{2+} channels; Ca^{2+} stores.

Introduction

Regulation of intracellular Ca^{2+} is intimately related to a number of cellular processes, including cell proliferation, differentiation, apoptosis, and cytotoxicity *(1–3)*. Intracellular Ca^{2+} homeostasis is maintained and changes in concentration of intracellular free Ca^{2+} ($[Ca^{2+}]_i$) are regulated through Ca^{2+} channels and pumps located in outer and inner cellular membranes, and through intracellular Ca^{2+} buffers (reviewed in *4*). Of particular interest, the steroid hormone 1,25-dihydroxyvitamin D_3[1,25$(OH)_2D_3$] regulates Ca^{2+} binding proteins calbindins, Ca^{2+} channels, and Ca^{2+}-ATPases *(4–7)* and inhibits growth of human breast cancer cells *(8,9)*.

Surprisingly, little is known about regulation of $[Ca^{2+}]_i$ in cancer cells, particularly breast cancer cells. Pathways for Ca^{2+} entry from extracellular space and Ca^{2+} mobilization from intracellular stores in breast cancer cells have not been described, yet mechanisms of action of major classes of antibreast cancer drugs: antiestrogens, alkylating agents, and topoisomerase II inhibitors, are linked to the Ca^{2+} signaling pathway *(10,11)*. Of special interest, tamoxifen, a classic therapeutic/chemopreventive agent for breast cancer, was recently shown to interact with targets implicated in Ca^{2+} homeostasis *(12,13)*, particularly, voltage-dependent Ca^{2+} channels (VDCC) *(14)*. Conflicting results regarding efficacy of intracellular Ca^{2+} modulating agents in breast cancer have been reported. Antagonists of VDCC

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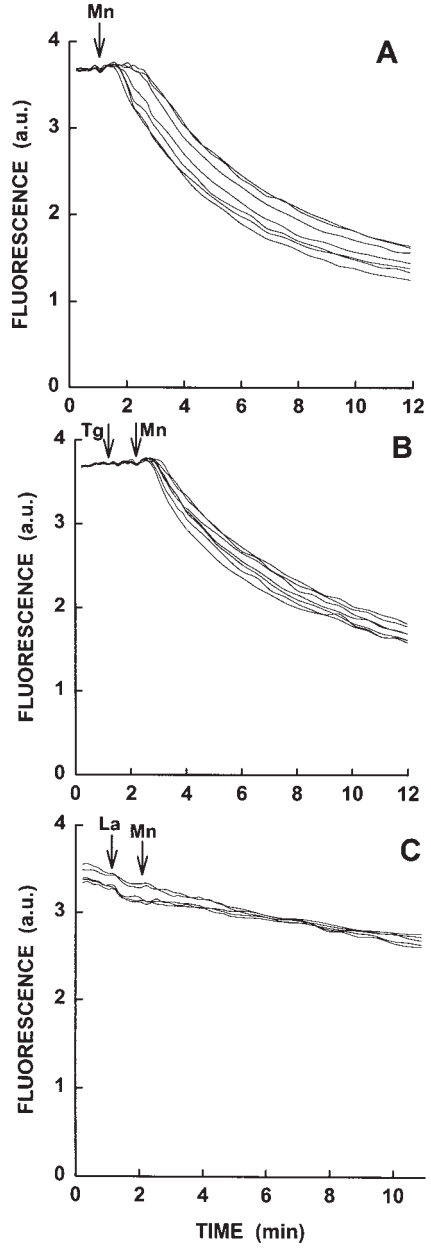

Fig. 1. Ca^{2+} entry through VICC. **(A)** BT-20 cells exhibited a pathway for Ca^{2+} influx that is permeant to Mn^{2+} ions, which quench fura-2 fluorescence. **(B)** Thapsigargin (Tg; 2 μ*M*) added before Mn^{2+}-containing solution, did not increase the rate of fluorescence quench by Mn^{2+} and, thus, of Ca^{2+} influx. **(C)** La^{3+} (0.5 m*M*), added before Mn^{2+}-containing solution, prevented fura-2 fluorescence quench by Mn^{2+}, which indicates blockage of Ca^{2+} entry into BT-20 cells. Here and in Figs. 2–5, chemicals were added to the bath solution where indicated by arrows; for each experimental situation, 1–3 cultures were used, and the traces represent responses of individual cells from the same culture.

were effective in suppressing growth of breast cancer cells in vitro and breast tumor progression in vivo *(15)*, and they circumvented multiple anticancer drug resistance *(16)*. In contrast, an epidemiological study revealed an increased breast carcinoma risk in postmenopausal women using Ca^{2+} channel blockers *(17)*.

The purpose of this study was to investigate pathways of Ca^{2+} entry and Ca^{2+} mobilization and their regulation by $1,25(OH)_2D_3$ in the human breast cancer cell line BT-20. This cell line does not express the estrogen receptor, but does express the vitamin D receptor *(18)*, implying that effects of $1,25(OH)_2D_3$ on $[Ca^{2+}]_i$ may be evaluated without considering possible interactions between the vitamin D- and estrogen-mediated signaling pathways. We employed in situ video imaging of fura-2-loaded cells to measure $[Ca^{2+}]_i$ simultaneously in individual cells under identical conditions. We describe the mechanisms of regulation of $[Ca^{2+}]_i$ in human breast cancer cells and the role of $1,25(OH)_2D_3$ in such regulation. The results of this study indicate that $[Ca^{2+}]_i$ regulatory mechanisms in breast cancer cells are somewhat limited and that antiproliferative actions of $1,25(OH)_2D_3$ may be related to its effects on $[Ca^{2+}]_i$.

Results

Ca^{2+} Entry Pathways

Ca^{2+} typically enters the cell through voltage-insensitive Ca^{2+} channels (VICC) and VDCC. The Ca^{2+} entry through nonselective VICC may be estimated by Mn^{2+} entry and fura-2 fluorescence quench *(19)*. Figure 1A shows that BT-20 cells express Ca^{2+} channels permeable to Mn^{2+}, i.e., nonselective VICC. The emptying of intracellular Ca^{2+} stores with thapsigargin (Fig. 1B) did not increase influx of extracellular Ca^{2+}, as estimated from the tangent of Mn^{2+} quench curves ($\tan\alpha = 0.36 \pm 0.016$ vs 0.37 ± 0.021 in controls, $n = 14$ cells). This suggests no significant activation of capacitative Ca^{2+} entry. La^{3+} and Ni^{2+} blocked Ca^{2+} influx, as estimated with Mn^{2+} quench technique (Fig. 1C). Raising the extracellular Ca^{2+} concentration evoked a rapid (within 1 min) and sustained (at least for several minutes) increase in $[Ca^{2+}]_i$ (216 ± 3.2 n*M* vs 139 ± 3.6 n*M* of the baseline $[Ca^{2+}]_i$, $p < 0.05$; *see* Fig. 5A). Collectively, these data indicate that breast cancer cells express nonselective VICC, but suggest that the channels are not depletion-operated and, thus, do not mediate capacitative Ca^{2+} entry.

Depolarization of BT-20 cells with high K^+ decreased Ca^{2+} entry (Fig. 2A) and slightly lowered basal $[Ca^{2+}]_i$, whereas Bay K8644, an agonist of L-type VDCC, had no effect on $[Ca^{2+}]_i$ (Fig. 2B). These results imply that VDCC are absent, but are consistent with expression of VICC in breast cancer cells.

Ca^{2+} Mobilization Pathways

Endoplasmic reticulum, accumulating Ca^{2+} at high concentrations, is an intracellular source of this signaling ion. Thapsigargin, a specific mobilizer of endoplasmic reticulum Ca^{2+} stores *(20)*, evoked a transient increase in $[Ca^{2+}]_i$ that returned relatively slowly to baseline (Fig. 3A). Removal of extracellular Ca^{2+} (Fig. 3B) decreased peak values of the Ca^{2+} mobilization response to thapsigargin (276 ± 12 n*M* vs 400 ± 23 n*M* in Ca^{2+}-containing solution,

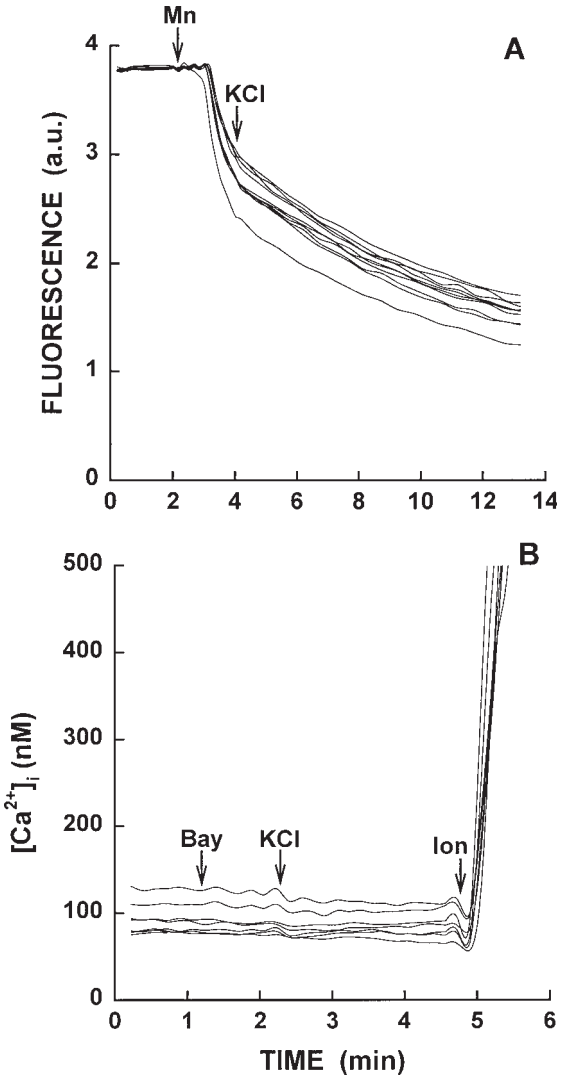

Fig. 2. Absence of VDCC. **(A)** High K^+ (70 m*M*) decreased the rate of Ca^{2+} entry and **(B)** slightly lowered basal $[Ca^{2+}]_i$ in some cells; Bay K8644 (5 μ*M*) did not increase $[Ca^{2+}]_i$. In depolarizing solution, isomolar NaCl was replaced for KCl.

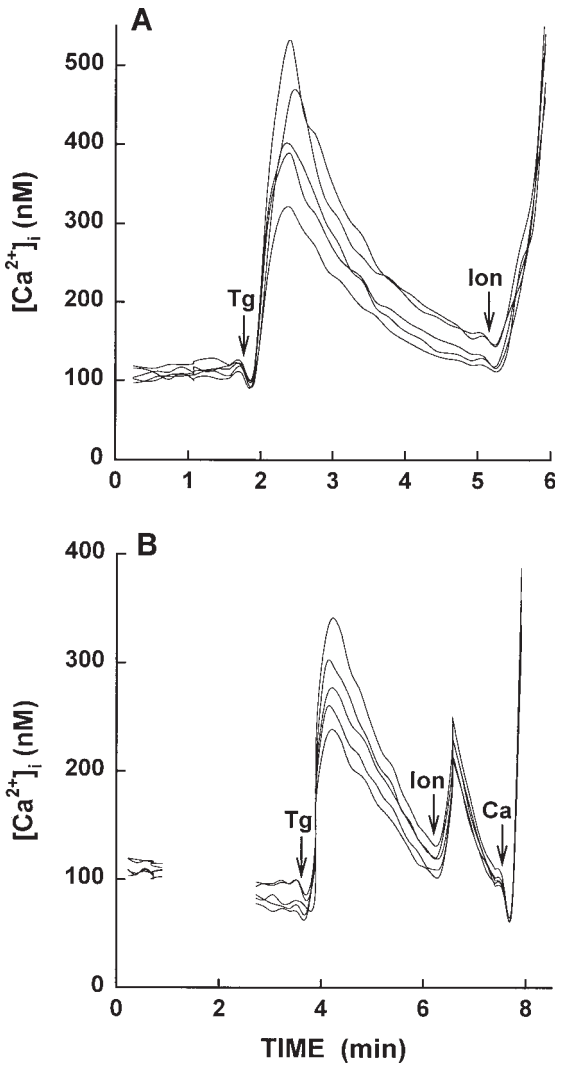

Fig. 3. Thapsigargin-sensitive Ca^{2+} stores. Thapsigargin (2 μ*M*) mobilized endoplasmic reticulum Ca^{2+} stores in Ca^{2+}-containing **(A)** and Ca^{2+}-free **(B)** buffer; subsequent addition of ionomycin (5 μ*M*) in Ca^{2+}-free buffer (*see* B) evoked only a small transient response. During the washout period (no traces), Ca^{2+}-containing buffer was replaced with "zero" Ca^{2+} solution containing 50 μ*M* EGTA.

$p < 0.05$, $n = 10$ cells), implying partial depletion of endoplasmic reticulum Ca^{2+} stores under these conditions. A Ca^{2+} ionophore ionomycin, added to thapsigargin-treated cells in nominally Ca^{2+}-free buffer, evoked only a small transient increase in $[Ca^{2+}]_i$ (198 ± 7.6 n*M*), as compared with a very large (>1000 n*M*) increase with extracellular Ca^{2+} present (compare Fig. 3A and B). An addition of Ca^{2+} induced a transient Ca^{2+} response in cells maintained in Ca^{2+}-free buffer and treated with thapsigargin (*see* right side of Fig. 5A), implying that the elevated extracellular Ca^{2+}-induced increase in $[Ca^{2+}]_i$ resulted from both Ca^{2+} mobilization from thapsigargin-sensitive endoplasmic reticulum Ca^{2+} stores and Ca^{2+} influx through VICC. Caffeine (40 m*M*), an agonist of the ryanodine receptor/Ca^{2+} release channel, did not induce Ca^{2+} release from intracellular stores, and the thapsigargin-sensitive Ca^{2+} pool remained intact (data not shown). These result demonstrate that rapidly mobilizable Ca^{2+} is stored principally in the endoplasmic reticulum of breast cancer cells.

Effects of 1,25(OH)$_2$D$_3$

As we and others have shown (reviewed in *4*), 1,25 $(OH)_2D_3$ can trigger a rapid increase in $[Ca^{2+}]_i$ in diverse cell types. This results from release of intracellular Ca^{2+} stores and/or influx of extracellular Ca^{2+}. However, we were not able to detect an increase in $[Ca^{2+}]_i$ in BT-20 cells up to 30 min after addition of 1,25$(OH)_2D_3$ (1–500 n*M*) (data not shown). Nevertheless, 1,25$(OH)_2D_3$ at a concentration of 100 n*M* induced an initial rapid (within 1 min) 1.7-fold increase in Ca^{2+} influx through VICC, as estimated by the rate of fura-2 fluorescence quench by Mn^{2+} ($\tan\alpha$ = 0.63 ± 0.018 vs 0.37 ± 0.021, $p < 0.05$, $n = 14$ cells;

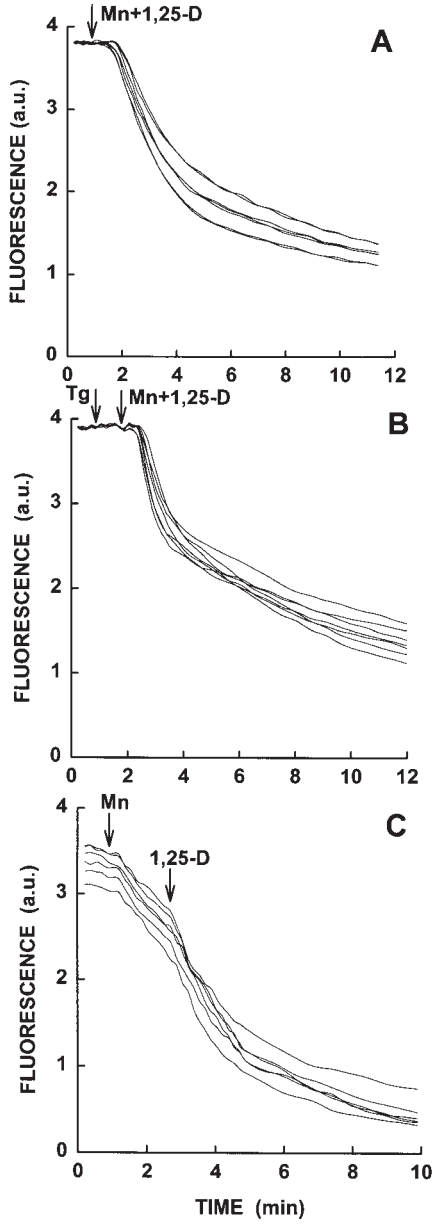

Fig. 4. $1,25(OH)_2D_3$ effects on VICC. **(A)** $1,25(OH)_2D_3$ (100 n*M*) increased the rate of Ca^{2+} influx within 1 min (compare with Fig. 1A). **(B)** Pretreatment with thapsigargin (2 μ*M*) did not further increase the rate of Mn^{2+} quench (compare with panel A of the figure). **(C)** Addition of $1,25(OH)_2D_3$ (100 n*M*) to the same cells after addition of Mn^{2+}-containing solution increased the rate of fura-2 fluorescence quench by Mn^{2+} and, thus, Ca^{2+} influx.

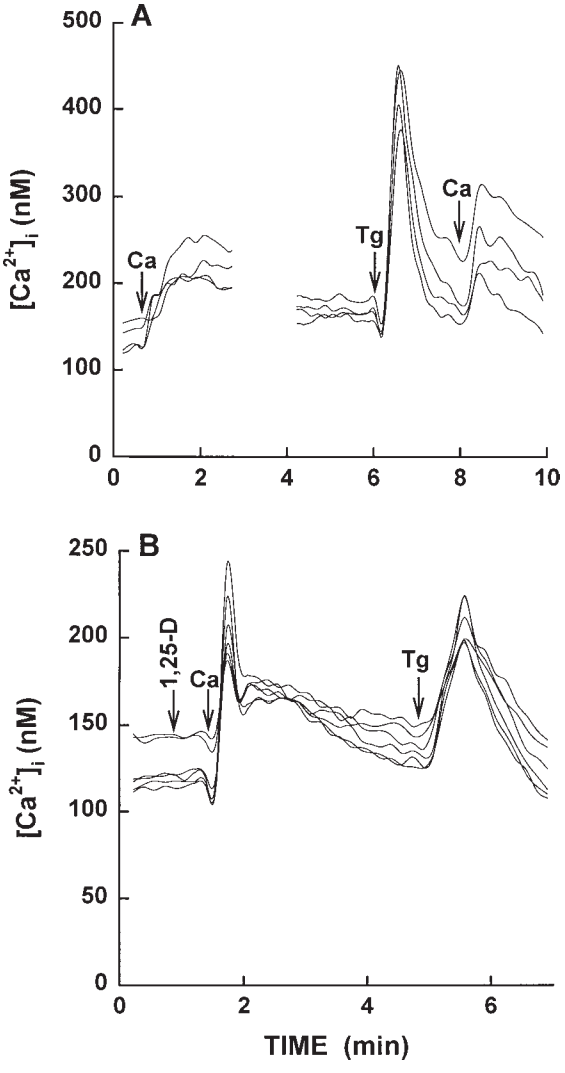

Fig. 5. Rapid effects of $1,25(OH)_2D_3$ and elevated extracellular Ca^{2+} on $[Ca^{2+}]_i$. **(A)** Increase in extracellular Ca^{2+} concentration (from 1 to 5 m*M*) evoked a sustained increase in $[Ca^{2+}]_i$; subsequent addition of thapsigargin (2 μ*M*) in Ca^{2+}-free buffer depleted Ca^{2+} stores, following which an increase in extracellular Ca^{2+} (5 m*M*) evoked a transient Ca^{2+} response. The gap in the traces indicates the washout period. **(B)** $1,25(OH)_2D_3$ (100 n*M*) appeared to sensitize Ca^{2+} stores so that an increase in concentration of extracellular Ca^{2+} produced a rapid $[Ca^{2+}]_i$ increase followed by a sustained phase. Subsequent addition of thapsigargin (2 μ*M*) showed that endoplasmic reticulum Ca^{2+} stores were partially depleted by $1,25(OH)_2D_3$.

also compare Fig. 4A with Fig. 1A). Addition of 1,25 $(OH)_2D_3$ to the same cells after addition of Mn^{2+} (Fig. 4C) significantly accelerated fura-2 fluorescence quench (tanα increased from 0.47 ± 0.011 to 0.73 ± 0.026, $p < 0.05$, $n = 12$ cells). The $1,25(OH)_2D_3$-evoked increase in Ca^{2+} influx was not affected by pretreatment of the cells with thapsigargin ($\tan\alpha = 0.65 \pm 0.016$ vs 0.63 ± 0.018 in corresponding controls, $n = 14$ cells; also compare Fig. 4B with Fig. 4A). Moreover, acute addition of $1,25(OH)_2D_3$ modified the $[Ca^{2+}]_i$ response to an increasing concentration of extracellular Ca^{2+}, so that a transient Ca^{2+} peak, probably reflecting Ca^{2+} release from the endoplasmic reticulum stores or rapid Ca^{2+} influx, preceded a sustained phase of $[Ca^{2+}]_i$ increase (Fig. 5B). A short rapid burst of recovery in $[Ca^{2+}]_i$, seen in this figure, may result from activation of a plasma membrane Ca^{2+}-ATPase by $1,25(OH)_2D_3$.

Treatment of serum-supplemented cultures with 1,25 $(OH)_2D_3$ (100 n*M*) for 4–72 h significantly increased basal $[Ca^{2+}]_i$ and the percentage of cells with elevated (>200 n*M*)

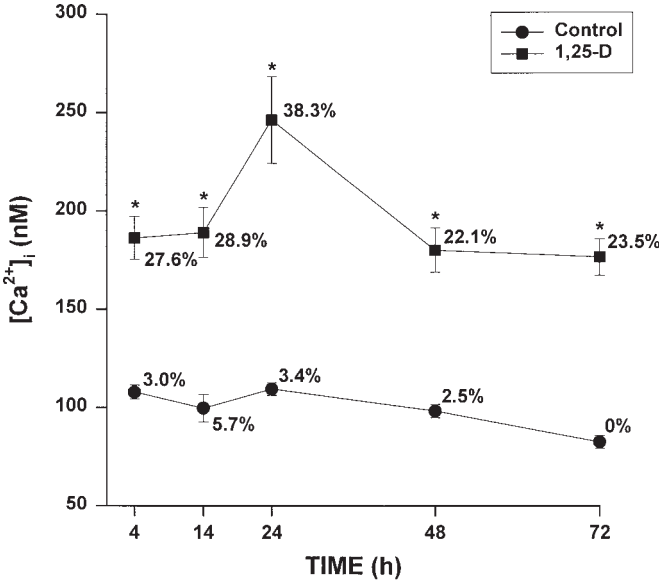

Fig. 6. Chronic effects of $1,25(OH)_2D_3$ on $[Ca^{2+}]_i$. Treatment of BT-20 cells with $1,25(OH)_2D_3$ (100 n*M*) for 4–72 h increased basal $[Ca^{2+}]_i$ and the percentage of cells with $[Ca^{2+}]_i$ > 200 n*M*. The data, analyzed by ANOVA, represent mean values ± SEM; $*p < 0.05$, as compared with corresponding control groups. The arbitrary $[Ca^{2+}]_i$ of 200 n*M* was selected because the percentage of control cells with this $[Ca^{2+}]_i$ was negligible. Therefore, this level may indicate the threshold for overcoming regulation of basal $[Ca^{2+}]_i$ by $1,25(OH)_2D_3$.

$[Ca^{2+}]_i$ (Fig. 6). Both the basal $[Ca^{2+}]_i$ and the percentage of cells with elevated $[Ca^{2+}]_i$ were highest after 24 h of exposure to $1,25(OH)_2D_3$ (*see* Fig. 6). Ca^{2+} responses of cells with an elevated basal $[Ca^{2+}]_i$ to thapsigargin were dramatically attenuated or abolished (data not shown). Taken together, these findings indicate that effects of $1,25(OH)_2D_3$ on $[Ca^{2+}]_i$ in breast cancer cells are primarily owing to mobilization of thapsigargin-sensitive endoplasmic reticulum Ca^{2+} stores.

Discussion

Recent studies indicate that intracellular Ca^{2+} regulates many key steps, from initiating early signaling events to causing irreversible changes, in the pathways leading to apoptosis, cytotoxicity, proliferation, and differentiation. Precise molecular mechanisms of how Ca^{2+} exerts these effects are not known, and Ca^{2+}-dependent targets have not been conclusively identified *(3,4)*.

Regulation of $[Ca^{2+}]_i$ underlies the temporal and spatial patterns of Ca^{2+} signaling, and maintaining intracellular Ca^{2+} homeostasis is crucial for genomic stability and functioning of the cell *(4)*. Thus, understanding mechanisms of cellular Ca^{2+} signaling and homeostasis in breast cancer may help in understanding the process of carcinogenesis and the development of improved strategies for treatment of this disease.

We employed fluorescence digital ratiomentric *in situ* video imaging with the Ca^{2+} indicator fura-2 to measure, at the single-cell level, $[Ca^{2+}]_i$ and Ca^{2+} responses in the human breast cancer cell line BT-20. Our findings provide a relatively complete picture of how these cells regulate $[Ca^{2+}]_i$. The major pathway for Ca^{2+} entry in BT-20 cells appears to be nonselective VICC. This type of Ca^{2+} channel can be coupled to plasmalemmal receptors and activated by second messengers *(21)*. As evaluated by the rate of Ca^{2+} entry (*see* Fig. 1), VICC in these breast cancer cells were not stimulated by depletion of intracellular Ca^{2+} stores, indicating that the capacitative Ca^{2+} entry pathway is not operable in these cells. In the absence of capacitative Ca^{2+} entry, a slow decrease phase in Ca^{2+} response to an inhibitor of the endoplasmic reticulum Ca^{2+}-ATPase thapsigargin (*see* Fig. 3) may indicate low efficiency of Ca^{2+} extrusion mechanisms and/or Ca^{2+} entry through an unknown pathway. However, the latter seems unlikely, because the temporal pattern and magnitude of Ca^{2+} responses to thapsigargin were similar for the cells in Ca^{2+}-containing and Ca^{2+}-free solutions (compare Fig. 3A and B).

The steroid hormone $1,25(OH)_2D_3$ triggered opening of VICC in breast cancer cells (*see* Fig. 4), possibly via induction of one or more second messengers *(4)*. However, the $1,25(OH)_2D_3$- stimulated influx of extracellular Ca^{2+} did not evoke a rapid increase in $[Ca^{2+}]_i$, probably owing to low conductance of VICC. We *(22)* and Vandewalle et al. *(23)* were also unable to demonstrate any early rise in $[Ca^{2+}]_i$ in MCF-7 breast cancer cells after application of $1,25(OH)_2D_3$. Moreover, our results show that BT-20 cells do not express the voltage-dependent pathway for Ca^{2+} entry (*see* Fig. 2). It is therefore unlikely that as Fitzpatrick et al. *(17)* suggested, Ca^{2+} channel blockers may interfere with Ca^{2+}-triggered apoptosis in human breast tumors and, hence, serve as tumor promoters.

The major Ca^{2+} storage compartment in BT-20 cells was endoplasmic reticulum. After blockage of the endoplasmic reticulum Ca^{2+}-ATPase with thapsigargin and depletion of these Ca^{2+} stores, a Ca^{2+} ionophore ionomycin mobilized only a small additional Ca^{2+} pool (*see* Fig. 3B). This clearly indicates that these breast cancer cells do not accumulate substantial quantities of Ca^{2+} in other compartments, e.g., mitochondria. Regulated release of Ca^{2+} from the endoplasmic reticulum stores most probably occurs through the inositol 1,4,5-trisphosphate (IP_3) receptor, because the ryanodine receptor/Ca^{2+} release channel appears to be absent in breast cancer cells (*see* Results). In this context, effects of $1,25(OH)_2D_3$ on $[Ca^{2+}]_i$ may result from both a rapid activation of Ca^{2+} entry through VICC and a delayed, sustained activation of IP_3 receptors via IP_3- and/or Ca^{2+}-mediated mechanisms *(24)*. Upregulation of IP_3 receptors by $1,25(OH)_2D_3$ has been reported *(25)*. Moreover, insufficient Ca^{2+} buffering by cytosolic Ca^{2+} binding proteins in breast cancer cells may contribute to the $1,25(OH)_2D_3$-induced sustained increase in $[Ca^{2+}]_i$ *(4)*.

In conclusion, breast cancer cells appear to have cellular Ca^{2+} homeostatic mechanisms limited to VICC, as related to Ca^{2+} entry pathways, and endoplasmic reticulum, as related to Ca^{2+} stores. The steroid hormone $1,25(OH)_2D_3$ at pharmacological concentrations is able to utilize those regulatory mechanisms and induce a marked, sustained increase in $[Ca^{2+}]_i$ in breast cancer cells. Such deregulation of $[Ca^{2+}]_i$ may dramatically affect cell fate, e.g., lead to the Ca^{2+}-mediated apoptotic cell death *(4)*.

Materials and Methods

Cell Culture

An established, estrogen receptor-negative and vitamin D receptor-positive human breast cancer cell line BT-20 was used in this study. BT-20 cells were obtained from American Type Culture Collection (Rockville, MD). Cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum and standard antibiotics at 37°C in a humidified atmosphere of 5% CO_2 in air. For $[Ca^{2+}]_i$ measurements, cells were plated on round glass cover slips and allowed to grow for 2–4 d (40–60% confluency).

Fura-2 Loading and Cell Handling

Cells on cover slips were loaded with 5 μ*M* fura-2/AM (cell-permeant acetoxymethyl ester) in Dulbecco's PBS (D-PBS) supplemented with 0.5% dimethylsulfoxide and 0.05% Pluronic F-127 at 37°C for 40 min. After incubation with fura-2/AM, the cells were maintained in D-PBS for a 10-min recovery period (to allow the intracellular esterases to cleave the dye completely to the impermeant form) and used within 30 min after the end of this period.

Fura-2-loaded cells were placed in a standard balanced salt solution (BSS) containing 1.0 m*M* $CaCl_2$ *(19)* in a microscope stage, temperature-controlled (37 ± 0.02°C) microincubation chamber with a bottom made of the cover slip. During the recording, the addition of chemicals and drugs were made either as 1000-fold concentrated solutions to achieve the required final concentration, or by aspiration and addition of fresh bathing solution with the compound to be tested. The concentration of vehicles, ethanol or dimethylsulfoxide, never exceeded 0.2%, and the vehicles did not affect $[Ca^{2+}]_i$ at this concentration.

Video Imaging of Intracellular Ca^{2+}

The dynamics of $[Ca^{2+}]_i$ were assessed with cells in the microincubation chamber on a Nikon Diaphot inverted microscope equipped for fluorescence digital ratiomentric video imaging (Fryer Co., Huntley, IL), as previously described *(19,26)*. Briefly, the images were captured using a Nikon Fluor ×40, 1.30 numerical aperture oil-immersion objective, averaged (16 video frames) and ratioed (340:380 nm excitation, 510 nm emission) on a pixel-by-pixel basis, and stored for analysis. A pair of images used for a "ratioed" image was collected every 5 s.

The fluorescence signal of $[Ca^{2+}]_i$ was calibrated at the end of experiments. Ca^{2+} saturation was achieved by adding 5 μ*M* ionomycin in the presence of 5 m*M* $CaCl_2$, and virtually zero $[Ca^{2+}]_i$ by further addition of 10 m*M* EGTA; $MnCl_2$ (50 m*M*) was subsequently added to obtain background fluorescence.

To measure the Mn^{2+} entry rate as a reporter of indirect Ca^{2+} influx *(19)*, the images were recorded at excitation of 360 nm (the fura-2 isosbestic point) and 2 m*M* of extracellular Mn^{2+}. The rates of Ca^{2+} entry were estimated from the tangent of the slope of the first ("rapid") portion of Mn^{2+} quench curves.

MetaFluor software (Universal Umaging, West Chester, PA) was used for image analysis. A mouse-driven pointer was employed to define, on the phase-contrast or fluorescent image, individual cells. Background correction was applied during data analysis.

Materials

Fura-2/AM, Pluronic F-127, and thapsigargin were purchased from Molecular Probes (Eugene, OR); Bay K8644 was obtained from Calbiochem (La Jolla, CA); other chemicals were purchased from Sigma (St. Louis, MO). Cell-culture medium and reagents were purchased from Gibco-BRL (Gaithersburg, MD).

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