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ORIGINAL ARTICLE

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Fendiline, an anti-anginal drug, increases intracellular Ca^{2+} in PC3 human prostate cancer cells

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Abstract *Background*: The effects of the anti-anginal drug fendiline on intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) in human PC3 prostate cancer cells were examined. *Methods*: $[Ca^{2+}]_i$ was measured using the fluorescent dye fura-2. *Results*: Fendiline (0.5–100 μ*M*) increased $[Ca^{2+}]_i$ in a concentration-dependent manner. Ca^{2+} removal partly inhibited the Ca^{2+} signals. In Ca^{2+}-free medium, pretreatment with 100 μ*M* fendiline inhibited most of the $[Ca^{2+}]_i$ increase induced by 1 μ*M* thapsigargin (an endoplasmic reticulum Ca^{2+} pump inhibitor), and pretreatment with thapsigargin abolished the fendiline-induced $[Ca^{2+}]_i$ increases. Adding 3 m*M* Ca^{2+} increased $[Ca^{2+}]_i$ in cells pretreated with 0.5–200 μ*M* fendiline in Ca^{2+}-free medium. Pretreatment with 1 μ*M* U73122 to block the formation of inositol-1,4,5-trisphosphate (IP_3) did not alter fendiline-induced internal Ca^{2+} release. *Conclusions*: The anti-anginal drug fendiline induced internal Ca^{2+} release and external Ca^{2+} entry. Because prolonged increases in $[Ca^{2+}]_i$ may lead to cell injury and death, the long-term effect of fendiline on the function of prostate cancer cells should be investigated.

Keywords Prostate cancer cells · PC3 · Fendiline · Fura-2 · Ca^{2+} signaling

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Introduction

Cellular Ca^{2+} is an important second messenger for growth regulation in cells [2, 3, 4]. Various agents have been shown to increase cytosolic free Ca^{2+} concentrations ($[Ca^{2+}]_i$) in PC3 androgen-independent human prostate cancer cells, including bombesin, gastrin-releasing peptide, ATP/UTP, lysophosphatidic acid, thrombin, endothelin, and histamine [22]. Thapsigargin and analogues [5, 7, 12], and calcitonin [16] also have similar effects. These findings indicate that androgen-independent prostate tumor cell lines express multiple types of Ca^{2+}-mobilizing machinery capable of elevating $[Ca^{2+}]_i$. Ca^{2+}-sensitive cellular events may therefore contribute to the progression of prostate cancer.

Aberrant Ca^{2+} signaling is a central feature of malignant cells and a potential target for anticancer therapy [21]. Programmed cell death (apoptosis) is a new target for prostatic cancer therapy [11]. In PC3 cells, apoptosis has been shown to be coupled to an increase in $[Ca^{2+}]_i$ [13, 19]. Thus, thapsigargin analogues have been found to cause apoptosis of PC3 cells [5, 7, 12], inducers of apoptosis have been shown to activate a Ca^{2+}-permeable cation channel [9], and Ca^{2+} influx inhibitors that alter Ca^{2+}-sensitive signal transduction pathways have been shown to suppress the proliferative and metastatic potential of PC3 cells [21].

Fendiline is an anti-anginal drug used in the treatment of coronary heart diseases [1]. In vitro, fendiline inhibits L-type Ca^{2+} channels [14, 20] and calmodulin

[1]. Notably, fendiline has been recently shown to act as a Ca^{2+} mobilizer in renal tubular cells by releasing stored Ca^{2+} and activating external Ca^{2+} influx [10]. Thus, in an attempt to search for new antiprostatic cancer drugs, in the present study the effects of fendiline on $[Ca^{2+}]_i$ in PC3 cells were investigated.

It was found using fura-2 as a Ca^{2+} probe that fendiline caused significant increases in $[Ca^{2+}]_i$ in PC3 cells. A concentration-response relationship was established, and the underlying mechanisms of the fendiline response determined.

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 humidified air containing 5% CO_2.

Solutions

Ca^{2+} medium (pH 7.4) contained 140 m*M* NaCl, 5 m*M* KCl, 1 m*M* $MgCl_2$, 1.8 m*M* $CaCl_2$, 10 m*M* Hepes, and 5 m*M* glucose. Ca^{2+}-free medium contained no added Ca^{2+} plus 1 m*M* EGTA. The experimental solution contained less than 0.1% 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 μ*M*) 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 medium and 0.5×10^6 cells. Fluorescence was monitored using a Shimadzu RF1503PC spectrofluorophotometer by recording excitation signals at 340 and 380 nm and the emission signal at 510 nm at 1-s intervals. Maximum and minimum fluorescence values were obtained by adding 0.1% Triton X-100 and 20 m*M* EGTA sequentially at the end of each experiment. $[Ca^{2+}]_i$ was calculated as described previously [8].

Chemical reagents

The reagents for cell culture were from Gibco (Grand Island, N.Y.). Fura-2/AM was from Molecular Probes (Eugene, Ore.). Fendiline was from RBI (Natick, Mass.). All other reagents were from Sigma (St. Louis, Mo.).

Statistical analyses

The plots are representative of four or five similar responses. All values are presented as the means ± SEM from four or five experiments. Because the data from each experiment were the average of responses from 0.5×10^6 cells, the variation among experiments was small. This means that the mean ± SEM of four or five experiments was able to reveal significant differences. Statistical comparisons were carried out using Student's *t*-test, and significance was accepted for *P*-values <0.05.

Results

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

In the presence of external Ca^{2+}, fendiline (0.5–100 μ*M*) increased $[Ca^{2+}]_i$ in a concentration-dependent manner (Fig. 1A). The response induced by 200 μ*M* fendiline was similar to that induced by 100 μ*M*. At a concentration of 100 μ*M*, fendiline induced a $[Ca^{2+}]_i$ increase which reached a maximum 121 ± 3 s ($n=4$; $P=0.014$) later with a net value of 181 ± 12 n*M* ($n=4$; $P=0.023$), followed by a sustained phase. The rise in the Ca^{2+} signal was slower in response to lower concentrations of fendiline. The $[Ca^{2+}]_i$ increase induced by 50 μ*M* fendiline was not affected by 0.1 m*M* La^{3+}, 10 μ*M* Ni^{2+}, nifedipine, verapamil or diltiazem ($n=3$; not shown).

Effect of external Ca^{2+} removal on the fendiline response

External Ca^{2+} removal partly inhibited the fendiline-induced $[Ca^{2+}]_i$ increase (Fig. 1B). The concentration-response relationships of the fendiline-induced $[Ca^{2+}]_i$ increase in the presence and absence of external Ca^{2+} are shown in Fig. 1C. Ca^{2+} removal abolished the $[Ca^{2+}]_i$ increases induced by 0.5–5 μ*M* fendiline and partly inhibited the increases induced by 10–200 μ*M* fendiline ($n=5$; $P=0.012$).

Effect of fendiline on Ca^{2+} influx

Depletion of internal Ca^{2+} pools often triggers Ca^{2+} influx via capacitative Ca^{2+} entry [15]. Capacitative Ca^{2+} entry is usually measured by reintroduction of Ca^{2+} following depletion of internal Ca^{2+} stores with the tested agent in Ca^{2+}-free medium. In Ca^{2+}-free medium, after pretreatment with 10–100 μ*M* fendiline for 340 s, the addition of 3 m*M* $CaCl_2$ evoked an increase in $[Ca^{2+}]_i$ with a net maximum value of 150 ± 5 n*M* (traces a–c; $n=4$; $P=0.011$; Fig. 1B). Adding $CaCl_2$ alone induced only a small increase in $[Ca^{2+}]_i$ with a net maximum value of 23 ± 3 n*M* (trace d; $n=4$; $P=0.009$).

The internal source of the fendiline response

In Ca^{2+}-free medium, the addition of 1 μ*M* thapsigargin, an endoplasmic reticulum Ca^{2+} pump inhibitor [17], induced a significant increase in $[Ca^{2+}]_i$ with a net maximum value of 81 ± 4 n*M* ($n=4$; $P=0.025$; Fig. 2A). This suggests that thapsigargin induced the release of Ca^{2+} from the endoplasmic reticulum Ca^{2+} store. Fendiline (100 μ*M*) added subsequently failed to induce a significant increase in $[Ca^{2+}]_i$ ($n=4$; $P=0.08$). Conversely, pretreatment with 100 μ*M* fendiline for about

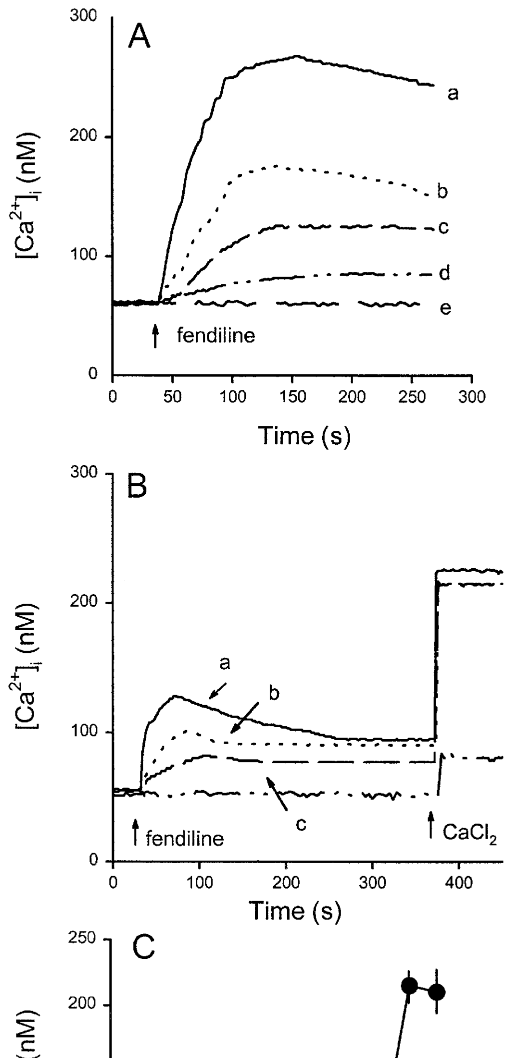

Fig. 1A–C Effects of fendiline on $[Ca^{2+}]_i$ in fura-2-loaded PC3 cells. **A** Fendiline-induced $[Ca^{2+}]_i$ increases in Ca^{2+} medium. The concentrations of fendiline were 100, 50, 10, 0.5 and 0 μM (*traces a, b, c, d, e*, respectively). **B** Effect of external Ca^{2+} removal on the fendiline-induced $[Ca^{2+}]_i$ increase and the effect of reintroduction of Ca^{2+}. Fendiline (0–100 μM) was added at 30 s to Ca^{2+}-free medium followed by the addition of 3 mM $CaCl_2$ at 380 s. The concentrations of fendiline were 100, 50, 10 and 0 μM (*traces a, b, c, d*, respectively). **C** Concentration-response plots of fendiline-induced Ca^{2+} signals in the presence (*circles*) and absence (*triangles*) of extracellular Ca^{2+}. The *y*-axis is the maximum value of the $[Ca^{2+}]_i$ response. Data are mean ± SEM from four or five experiments (*$P < 0.05$ between circles and triangles)

700 s abolished the thapsigargin-induced $[Ca^{2+}]_i$ increases ($n = 5$; $P = 0.013$).

Effect of blocking IP_3 formation on fendiline-induced Ca^{2+} release

Experiments were performed to determine whether fendiline induced release of internal Ca^{2+} via IP_3, by exploring the effect of inhibiting phospholipase C on fendiline-induced $[Ca^{2+}]_i$ increase. In Ca^{2+}-free medium 10 μM ATP, a well-established IP_3-coupled Ca^{2+} mobilizer, induced a transient rise in $[Ca^{2+}]_i$, with a net maximum value of 45 ± 3 nM ($n = 4$; $P = 0.015$; Fig. 3A), suggesting that PC3 cells possess IP_3-coupled Ca^{2+}-mobilizing machinery. Pretreatment with 1 μM U73122, a phospholipase C inhibitor [18], for 170 s abolished the 10 μM ATP-induced $[Ca^{2+}]_i$ increase ($n = 5$; $P = 0.018$; Fig. 3B). This most likely suggests that IP_3 formation was inhibited by U73122. However, 100 μM fendiline added at 290 s still induced an increase in $[Ca^{2+}]_i$ which was indistinguishable from the control fendiline response shown in Fig. 1B (trace a; without U73122 and ATP pretreatment; $n = 4$).

Discussion

This study presents the first attempt to examine the effect of fendiline, a clinically used anti-anginal drug, on cultured human prostate cancer cells. Fendiline caused a significant concentration-dependent increase in $[Ca^{2+}]_i$ starting at a concentration of 0.5 μM, and the response became saturated at 100 μM. In Ca^{2+} medium, the $[Ca^{2+}]_i$ increases induced by fendiline were sustained during incubation for 5 min. Because prolonged elevations in $[Ca^{2+}]_i$ are closely linked to cell dysfunction and apoptosis [2, 3, 4, 11], the effect of fendiline on $[Ca^{2+}]_i$ may lead to cytotoxicity.

External Ca^{2+} influx and internal Ca^{2+} release contributed to the Ca^{2+} signal induced by fendiline because external Ca^{2+} removal partly inhibited the signal. The Ca^{2+} store of fendiline-induced Ca^{2+} appeared to be the thapsigargin-sensitive endoplasmic reticulum pool because in Ca^{2+}-free medium, pretreatment with 1 μM thapsigargin abolished the fendiline-induced $[Ca^{2+}]_i$ increase and, vice versa, pretreatment with fendiline abolished the thapsigargin-induced $[Ca^{2+}]_i$ increase. This suggests that the endoplasmic reticulum is an important internal Ca^{2+} store in PC3 cells.

It appears that fendiline releases internal Ca^{2+} in a manner independent of IP_3 levels because when IP_3 formation was suppressed by inhibiting phospholipase C with 1 μM U73122, fendiline still released internal Ca^{2+} normally. It remains to be determined how fendiline releases Ca^{2+} from the endoplasmic reticulum.

Another question was how fendiline induces Ca^{2+} influx. The data in Fig. 2B suggest that in Ca^{2+}-free

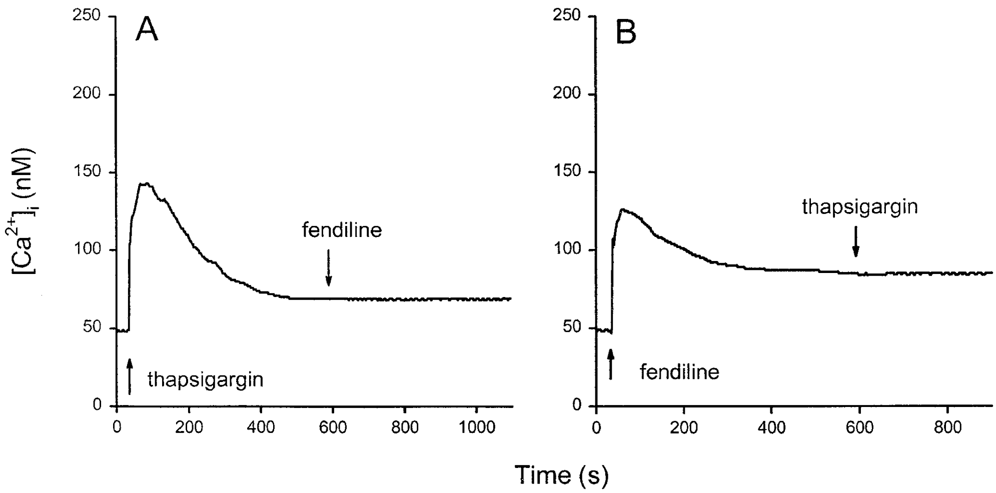

Fig. 2A, B Internal Ca^{2+} stores of fendiline-induced Ca^{2+} release. **A** Thapsigargin (1 μ*M*) and fendiline (100 μ*M*) were added to Ca^{2+}-free medium, as shown. **B** Similar to **A** except that the order of drug addition was reversed

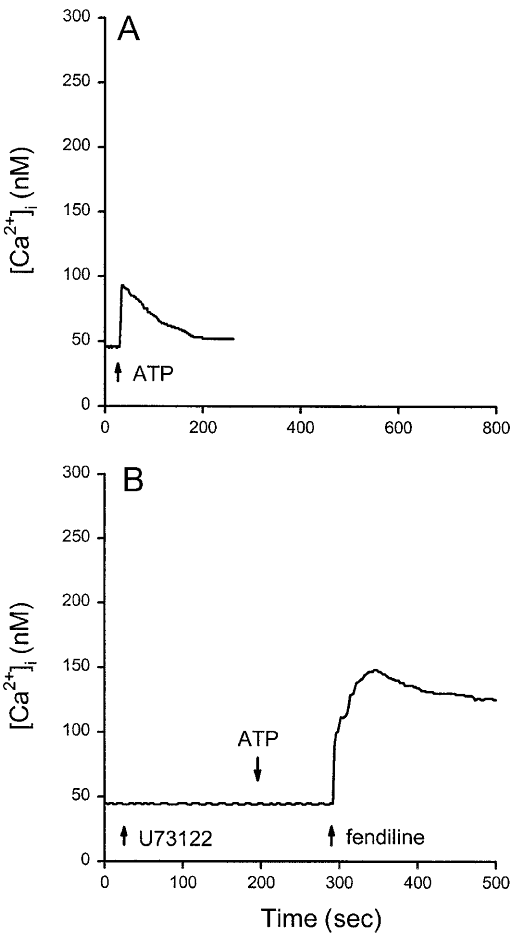

Fig. 3A, B Effect of inhibiting IP_3 formation on fendiline-induced $[Ca^{2+}]_i$ increase. **A** ATP (10 μ*M*) was added to Ca^{2+}-free medium at 30 s. **B** U73122 (2 μ*M*), ATP (10 μ*M*) and fendiline (100 μ*M*) were added to Ca^{2+}-free medium as shown

medium, after 5–200 μ*M* fendiline had depleted the Ca^{2+} stores for 5 min, the addition of Ca^{2+} induced an increase in $[Ca^{2+}]_i$ to a maximum that was about five-fold greater than control. This suggests that fendiline-induced Ca^{2+} influx could be via capacitative Ca^{2+} entry (a process controlled by Ca^{2+} store depletion), or direct opening of plasmalemmal Ca^{2+} channels.

Fendiline has been recently reported to increase $[Ca^{2+}]_i$ in a renal tubular cell line (MDCK) by releasing internal Ca^{2+} and activating external Ca^{2+} influx [10]. The effect of fendiline found in the present study was similar to that found in MDCK cells except for one striking difference. In MDCK cells, fendiline-induced external Ca^{2+} entry was inhibited by 0.1 m*M* La^{3+} by 50%, but in PC3 cells La^{3+} had no effect. This suggests that kidney cells and prostate cancer cells have different Ca^{2+} entry pathways.

The range of concentrations (0.5–100 μ*M*) at which fendiline has been found to increase $[Ca^{2+}]_i$ in nonexcitable cells such as renal cells and prostate cells is commonly used by researchers to inhibit voltage-gated Ca^{2+} channels in excitable cells. Fendiline has been found to inhibit the transient outward current in rat ventricular cardiomyocytes at 3 μ*M* [6], block L-type Ca^{2+} channels in rat ventricular myocytes at 1 μ*M* [14], and inhibit L-type Ca^{2+} channels in guinea-pig ventricular myocytes at 0.3–100 μ*M* [20]. Due to its blocking effect on L-type Ca^{2+} channels, fendiline is used as an anti-anginal drug. However, our results suggest that the Ca^{2+}-mobilizing effect of fendiline on nonexcitable cells should be taken into consideration in its clinical use.

The effect of fendiline on $[Ca^{2+}]_i$ in human prostate cancer cells was investigated in this study, and the underlying mechanisms were also examined. Because it has been shown that patients with angina taking fendiline orally may have a fendiline serum level of 0.6 μ*M* [23], our results may have clinical relevance.

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