Extracellular zinc and zinc-citrate, acting through a putative zinc-sensing receptor, regulate growth and survival of prostate cancer cells

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Prostate Zn2+ concentrations are among the highest in the body, and a marked decrease in the level of this ion is observed in prostate cancer. Extracellular Zn2+ is known to regulate cell survival and proliferation in numerous tissues. In spite of this, a signaling role for extracellular Zn2+ in prostate cancer has not been established. In the present study, we demonstrate that prostate metastatic cells are impermeable to Zn2+, but extracellular Zn2+ triggers a metabotropic Ca2+ rise that is also apparent in the presence of citrate. Employing fluorescent imaging, we measured this activity in androgen-insensitive metastatic human cell lines, PC-3 and DU-145, and in mouse prostate tumor TRAMP-C1 cells but not in androgen-sensitive LNCaP cells. The Ca2+ response was inhibited by Goq and phospholipase C (PLC) inhibitors as well as by intracellular Ca2+ store depletion, indicating that it is mediated by a Goq-coupled receptor that activates the inositol phosphate (IP3) pathway consistent with the previously identified zinc-sensing receptor (ZnR). Zn2+-dependent extracellular signal-regulated kinase and AKT activation, as well as enhanced Zn2+-dependent cell growth and survival, were observed in PC-3 cells that exhibit ZnR activity, but not in a ZnR activity-deficient PC-3 subtype. Interestingly, application of Zn2+-citrate (Zn2+Cit), at physiological concentrations, was followed by a profound functional desensitization of extracellular Zn2+-dependent signal transduction and attenuation of Zn2+-dependent cell growth. Our results indicate that extracellular Zn2+ and Zn2+Cit, by triggering or desensitizing ZnR activity, distinctly regulate prostate cancer cell growth. Thus, therapeutic strategies based either on Zn2+ chelation or administration of Zn2+Cit may be effective in attenuating prostate tumor growth.

Introduction

Zn2+ homeostasis in the prostate is more dynamic than in other human tissues, as prostate cells accumulate this ion and secrete it into the prostatic and seminal fluid. Zn2+ is secreted with citrate into the prostatic fluid (1), where it is found at high, i.e. millimolar, concentrations (2). The concentration of ‘free’ Zn2+ in this organ is, however, lower than these nominal values (3) since it is mostly complexed to citrate. The accumulation of Zn2+ in prostate cells is facilitated by the activity of Zn2+ transporters from the ZIP family, which mediate vectorial transport of this ion (4,5). By inhibiting mitochondrial aconitase, the major oxidizing enzyme of citrate, intracellular Zn2+ contributes to citrate accumulation in the prostate cells that is then secreted into the seminal fluid (6,7). The inhibition of mitochondrial aconitase significantly lowers adenosine triphosphate (ATP) production, leading to bioenergetic deficiency and attenuation of non-neoplastic prostate cell growth (2). It has been also shown that mitochondrial Zn2+ accumulation is involved in the apoptotic pathways in prostate epithelial cells (8,9). Thus, intracellular Zn2+ accumulation is expected to attenuate prostate cell growth.

That Zn2+ plays a role in prostate tumorigenesis is implied by the fact that Zn2+-accumulating cells were found primarily in the dorso-lateral lobe in a rat model, where prostate cancer is also most common in human (10,11). Furthermore, during prostate cancer development a 10-fold decrease in both Zn2+ and citrate concentrations are observed in the prostate epithelium (2). Decreased intracellular Zn2+ has been associated with reduced expression of the Zn2+ transporters, ZIP, that mediate influx of Zn2+ into prostate epithelial cells (12). It has been suggested that because of the lower Zn2+ concentration in malignant cells, ATP production is more efficient than in the non-malignant cells, thereby facilitating enhanced proliferation of the malignant cells. While metabolic and signaling roles for intracellular Zn2+ have been suggested (2,6,13), the possible contribution of extracellular Zn2+, or the Zn2+-citrate (Zn2+Cit) complex, to prostate cell growth is still unknown.

Extracellular Zn2+ promotes cell growth by activating and regulating major signaling pathways in numerous cell types. Zn2+, for example, activates the mitogen-activated protein kinase (MAPK) pathway, leading to enhanced proliferation of the colonocytic tumor cell line, HT-29, and NIH3T3 fibroblasts (14–16). Application of Zn2+ also activates the phosphoinositide-3 kinase (PI3K) in human bronchial epithelial cells (17,18) and leads to enhanced survival of fibroblasts (15,19) and neurons (20). Supplementation of Zn2+ also reduces the cellular accumulation of the tumor suppressor p53 in breast cancer cells (21).

Our previous work showed that extracellular Zn2+ induces cellular signaling via a G-protein-coupled receptor (GPCR), termed zinc-sensing receptor (ZnR) (22). We subsequently demonstrated Zn2+-sensing activity in a variety of epithelial cells (14,23) with affinity in the 10–100 μM range, i.e. within the range of its physiological concentration in those tissues. ZnR triggers activation of the inositol phosphate (IP3) pathway upon a rise in extracellular Zn2+. The subsequent Ca2+ release in HT-29 colonocytes activates extracellular signal-regulated kinase (ERK) and PI3K pathways and enhances the activity of the Na+/H+ exchanger, NHE1, which regulates cell volume and survival (14). Desensitization of GPCRs is also a protective mechanism against prolonged and potentially harmful activation by their ligands. The ZnR is functionally desensitized following exposure to extracellular Zn2+ (14,23). Desensitization of ZnR in HT-29 colonocytes abolished the Zn2+-dependent activation of the IP3 and the MAPK pathways. Thus, ZnR is emerging as a major link between changes in extracellular Zn2+ and signaling related to cell growth.

In the present study, we have examined the signaling role of extracellular Zn2+ in prostate cancer cells. We demonstrate that a rise in extracellular Zn2+ concentration induces ZnR activity and triggers signaling pathways that facilitate cell growth. Prolonged exposure to Zn2+ subsequently desensitizes ZnR activity, suggesting that in normal prostate tissue, where Zn2+ concentration is high, ZnR is inactive. Based on our data, we propose that in the neoplastic prostate and in peripheral tissues where metastatic cells encounter substantially lower Zn2+ concentrations, ZnR is activated by transient increases in Zn2+ concentration, thereby promoting prostate tumor cell growth.

Materials and methods

Cell culture

PC-3, DU-145, TRAMP-1 and LNCaP cells were obtained from the American Type Culture Collection; PC-3B was identified from a PC-3 line (American Type Culture Collection, Manassas, VA) cultured in the laboratory of Prof. Yossi Levy at Ben-Gurion University. All cells were cultured in RPMI 1640,

Abbreviations: ATP, adenosine triphosphate; CaEDTA, calcium ethylenediamine tetra-acetic acid; ERK, extracellular signal-regulated kinase; GPCR, G-protein-coupled receptor; IP3, inositol phosphate; MAPK, mitogen-activated protein kinase; PI3K, phosphoinositide-3 kinase; PKC, protein kinase C; TG, thapsigargin; ZnR, zinc-sensing receptor; Zn2+Cit, Zn2+–citrate.
supplemented with 10 mM (4-2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES), 1 mM sodium pyruvate, 10% fetal bovine serum, 4 mM l-glutamine and 0.5 μg/ml penicillin–streptomycin (24). Cells were seeded on glass coverslips, one day prior to fluorescent imaging experiments.

**Fluorescent calcium imaging**

Cells grown on coverslips were loaded for 30 min at room temperature with 5 μM Fura-2 AM (TEF Labs, Austin, TX) in Ringer’s solution (in millimolar): 120 NaCl, 5.4 KCl, 1.8 CaCl₂, 0.8 MgCl₂, 10 HEPES, 10 glucose, pH 7.4, containing 0.1% bovine serum albumin. Ca²⁺ imaging was carried out as described previously (22). The ratio of the fluorescent signal (R = F₅₀₀/F₃₈₀) was normalized to an initial baseline averaged over first 10 points acquired (R₀) and presented as percentage of the baseline (R/R₀ × 100). For Zn²⁺ imaging, cells were loaded as described with 5 μM Newport Green and the fluorescent signal was monitored using 488 nm wavelength for excitation (25). The fluorescent signal was normalized as described for Fura-2. In all experiments, representative graphs of 50–80 cells from at least three independent experiments are shown.

**Immunoblot analysis**

Cells were harvested immediately following treatment (unless otherwise indicated) into lysis buffer on ice in the presence of protease inhibitor mixture (Complete, Roche Applied Science, Mannheim, Germany) and centrifuged for 30 min (14,000 r.p.m.), as described previously (14). Supernatants (cytosolic fraction) were collected, sodium dodecyl sulfate sample buffer was added and samples were boiled for 5 min and then frozen at −80°C until used. Cell samples containing the cytosolic fraction (20 μg) were separated on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by immunoblotting. Antibodies against doubly phosphorylated ERK1/2 and total ERK1/2 or phosphorylated AKT and total AKT (Sigma, St. Louis, MO: Cell Signaling, Danvers, MA) were detected digitally using ChemiImager 5 (Alpha-Innotech, Israel Labtrade), and blots were quantified using this software. Phospho-ERK1/2 or AKT levels were normalized to total ERK1/2 or AKT protein, respectively. Phosphorylation of ERK1/2 or AKT is presented as percentage of the effect triggered by application of 100 μM Zn²⁺ unless otherwise indicated.

**Cell proliferation assay**

The quantitative sulphorhabdine B colorimetric assay (26) was used to determine cell proliferation. Cells (5000 cells per well) were seeded in 96-well plates. Cells were incubated daily (5 days) for 10 min in Ca²⁺-free Ringer’s solution supplemented with 100 μM ZnSO₄ or with the Zn²⁺ chelator calcium ethylenediamine tetra-acetic acid (CaEDTA) (100 μM). Cells were then reintroduced into RPMI medium containing 1% serum and CaEDTA. Zn²⁺ desensitization was induced by pretreating cells daily with 100 μM ZnSO₄ in Ca²⁺-containing Ringer’s solution for 60 min followed by 3× wash with Ca²⁺-free Ringer’s solution. Cells were then stained with sulphorhabdine B and optical density was measured at 492 and 620 nm using enzyme-linked immunoassorbent assay plate reader. Statistical analysis was performed on the averaged cell number (±SEM) of at least three independent experiments.

**Cell viability assay**

Cells (70 000) were seeded in six-well plates. Cells treated with Zn²⁺, or controls, were centrifuged to sediment all cells to the bottom of the plate and trypsin blue (0.4%, Sigma) was added (dilution 1:20). Three images were acquired from each plate, using an upright Olympus microscope (>10 objective) equipped with a SPOT digital camera. Cell counting was performed blind and averaged over three independent experiments.

**Statistical analysis**

Each bar graph represents an average ± SEM of at least three independent experiments. Statistical analysis was performed using paired Student’s t-test, comparing each treatment to addition of Zn²⁺ unless otherwise mentioned: *P < 0.05; **P < 0.01.

**Results**

Since previous studies have shown a decrease in the expression of the ZIP transporters and intracellular Zn²⁺ during prostate cancer (12), we sought to determine whether changes in Zn²⁺ concentration activate cellular signaling. We first monitored Zn²⁺ permeation rates in the androgen-insensitive, metastatic prostate cancer cell line, PC-3, using the intracellular Zn²⁺-specific dye, Newport Green. PC-3 cells loaded with Newport Green were monitored while superfusing the cells with Zn²⁺-containing Ringer’s solution (Figure 1A). No increase in fluorescence was monitored when Zn²⁺ (200 μM) was applied, whereas an increase in fluorescence was observed following addition of Zn²⁺ (200 μM) in the presence of the Zn²⁺ ionophore, pyrithione (5 μM). This indicates that PC-3 cells are highly impermeable to Zn²⁺ and is in agreement with previous studies (24,6,12,13).

We then asked if extracellular Zn²⁺ induces intracellular Ca²⁺ signaling. Changes in intracellular Ca²⁺ triggered by Zn²⁺ were monitored in PC-3 cells loaded with Fura-2. Application of 10–100 μM Zn²⁺ was followed by a rise in fluorescence that was already apparent with the addition of 10 μM Zn²⁺ and increased by 6-fold with 100 μM Zn²⁺ (Figure 1B). To determine if the Ca²⁺ rise resulted from Ca²⁺ influx from the extracellular medium or from Ca²⁺ released from intracellular pools, PC-3 cells were superfused with Ca²⁺-containing or Ca²⁺-free Ringer’s solution. The increase in fluorescence triggered by 100 μM Zn²⁺ was independent of extracellular Ca²⁺ (Figure 1C), indicating that it did not result from Ca²⁺ permeation. To further study the role of the intracellular Ca²⁺ stores, Zn²⁺ was applied after depletion of intracellular Ca²⁺ stores with the sarcoendoplasmic reticulum calcium ATPase inhibitor, thapsigargin (TG, 0.5 μM) and the purinergic P2Y agonist (uridine triphosphate, 100 μM) in Ca²⁺-free Ringer’s solution. Following store depletion, when cytoplasmic Ca²⁺ was at the basal level, application of Zn²⁺ failed to elicit an increase in Fura-2 fluorescence (Figure 1D). This indicates that the observed rise in fluorescence is related to Ca²⁺ release from TG-sensitive endoplasmic reticulum stores. Since Fura-2 is also a high affinity Zn²⁺ probe (Kₘ for Zn²⁺ ~2 nM), the lack of response following store depletion is a further proof that Zn²⁺ does not permeate into the cells.

We next asked if the IP₃ pathway mediates the Zn²⁺-dependent Ca²⁺ response in PC-3 cells, similar to the ZnR (22). Application of the phospholipase C (PLC) inhibitor, U73122 (1 μM), was followed by inhibition of the Zn²⁺-dependent Ca²⁺ response (Figure 1E). To assess the role of Goq in linking Zn²⁺ to activation of the IP₃ pathway, cells were incubated with a Goq inhibitor, YM-254890 (1 μM [27]), and were subsequently superfused with Zn²⁺. The Zn²⁺-dependent Ca²⁺ rise was completely inhibited by the application of YM-254890 (Figure 1F). Analysis of dose dependence of the Zn²⁺-dependent Ca²⁺ response yielded a Kₘ of 200 ± 35 μM for extracellular Zn²⁺ (supplementary Figure 1 is available at Carcinogenesis Online), indicating that although the ZnR response is strongly activated by low ‘free’ Zn²⁺ concentrations found in the prostate, it is activated by a very broad range of Zn²⁺ concentrations. Thus, our results strongly indicate that PC-3 cells possess a Goq-coupled Zn²⁺ sensing receptor that mediates activation of the IP₃ pathway to trigger intracellular Ca²⁺ release. A similar Zn²⁺-dependent signaling pathway, triggered by a putative ZnR, was described previously by us (14,22,23).

To determine if extracellular Zn²⁺-dependent signaling is also found in other prostate cancer cells, we studied Zn²⁺-dependent Ca²⁺ release in the androgen-insensitive human prostate cancer cells, DU-145, and in TRAMP-C1 cells, derived from mouse prostate tumor (28). In DU-145 cells, application of 300 μM Zn²⁺ triggered an intracellular Ca²⁺ rise (Figure 2A). Yet, application of Zn²⁺ (300 μM) following depletion of intracellular Ca²⁺ stores, using ATP (100 μM) and TG (1 μM) in Ca²⁺-free Ringer’s solution, failed to elicit an intracellular Ca²⁺ rise. In TRAMP-C1 cells, application of 100 μM Zn²⁺ triggered an intracellular Ca²⁺ rise (Figure 2B) that was diminished by the Goq inhibitor, YM-254890 (1 μM). Finally, application of 100 μM (data not shown) or 300 μM Zn²⁺ to the androgen-sensitive LNCaP prostate cancer cell line (Figure 2C) did not trigger a Ca²⁺ rise, although application of TG (1 μM) triggered a Ca²⁺ rise, suggesting that Ca²⁺ stores were not depleted. These results indicate that ZnR activity is triggered in the androgen-insensitive PC-3 and DU-145, as well as in TRAMP-C1 cells, but not in the androgen-sensitive LNCaP cells.

**Desensitization of Zn²⁺-dependent Ca²⁺ signaling**

We have previously demonstrated that the ZnR is prone to desensitization by prolonged exposure to Zn²⁺ (14,23). We therefore asked if desensitization plays a role in Zn²⁺-dependent signaling also in prostate cells. To test this, PC-3 cells were treated with 100 μM Zn²⁺ for 60 min in Ca²⁺-containing Ringer’s solution, washed in Zn²⁺-free Ringer’s (5 min) and subsequently Zn²⁺ was reapplied while...
monitoring intracellular Ca^{2+}. Pretreatment with Zn^{2+} resulted in complete inhibition of the Zn^{2+}-dependent Ca^{2+} response (Figure 3A), indicating that it is very effectively desensitized by Zn^{2+}. In the prostate, Zn^{2+} is present in very high concentrations (i.e. 6 mM), but is largely complexed with citrate (10 mM), which effectively lowers the concentration of free Zn^{2+} (3). It was of interest, therefore, to determine if Zn^{2+}Cit, in this physiological milieu, modulates intracellular Ca^{2+} signaling. We accomplished this by monitoring the Ca^{2+} response of PC-3 cells following application of Zn^{2+}Cit (6 mM Zn^{2+} and 10 mM citrate). As shown in Figure 3B, no intracellular Ca^{2+} rise was observed following application of Zn^{2+}Cit at these concentrations. The free Zn^{2+} concentration using this Zn^{2+}Cit ratio is estimated to be 20 μM (WebMax software), a concentration that is potentially sufficient to trigger Zn^{2+}-dependent signaling (see Figure 1B). Modulating the concentrations of Zn^{2+} and citrate, leading to a larger excess of free Zn^{2+}, resulted in a smaller Ca^{2+} response than that triggered by the same concentration of Zn^{2+} alone (Figure 3B and C). This suggests that the Zn^{2+}-dependent signaling was partially attenuated by Zn^{2+}Cit. We then asked if Zn^{2+}Cit, at physiological concentrations, will desensitize the Zn^{2+}-dependent Ca^{2+} response. The desensitization paradigm described for Zn^{2+} was performed using Zn^{2+}Cit at the physiological concentration (6 mM Zn^{2+} and 10 mM citrate). Pre-exposure of PC-3 cells to Zn^{2+}Cit was followed by profound attenuation of the Ca^{2+} response to extracellular application of 100 μM Zn^{2+} (Figure 3D). In contrast, when cells were preincubated with citrate (10 mM), in the absence of Zn^{2+}, subsequent application of Zn^{2+} triggered a robust Ca^{2+} response, thereby attesting to a specific role for Zn^{2+}Cit in desensitization of Zn^{2+}-dependent signaling in PC-3 cells (see Discussion).

**Role of Zn^{2+} in activation of MAPK and PI3K in prostate cancer cells**

We then sought to determine whether in Zn^{2+}-dependent PC-3 cells, extracellular Zn^{2+} regulates the MAPK and PI3K pathways linked to proliferation and survival of prostate cancer cells (29). Addition of 100 μM Zn^{2+} for 10 min to PC-3 cells induced phosphorylation of both ERK1/2 and AKT, whereas control cells treated with the

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**Fig. 1.** Extracellular Zn^{2+}-dependent intracellular Ca^{2+} release is mediated by the IP$_3$ pathway in the androgen-independent human prostate cancer cell line, PC-3. (A) PC-3 cells, loaded with Newport Green, were superfused with Ringer’s solution containing 200 μM Zn^{2+}, and subsequently Zn^{2+} (200 μM) was added in the presence of pyrithione (5 μM), at the times indicated by the arrows. (B) PC-3 cells, loaded with Fura-2, were superfused with Ringer’s solution and 10–100 μM Zn^{2+} were applied at the indicated time (marked by the arrow). (C) PC-3 cells were superfused with Ca^{2+} free or Ca^{2+}-containing Ringer’s solution and Zn^{2+} was applied as indicated (arrow). (D) PC-3 cells were treated with the sarcoendoplasmic reticulum calcium ATPase inhibitor TG (0.5 μM) and the purinergic P2Y agonist UTP (uridine triphosphate, 100 μM) in Ca^{2+}-free Ringer’s to induce depletion of the Ca^{2+} stores. Subsequently Zn^{2+} was added. (E) Zn^{2+} was added to PC-3 cells preincubated with the PLC inhibitor U73122 (1 μM). (F) Zn^{2+} was applied to PC-3 cells and the Ca^{2+} response was observed, and Zn^{2+} was reapplied in the presence of the Gq inhibitor, YM-254890 (1 μM). A response to reapplication of Zn^{2+}, at the same concentrations, in control PC-3 cells is shown in the insert.
extracellular Zn\(^{2+}\) chelator, CaEDTA (100 μM), exhibited very low phosphorylation (Figure 4A and B). No Zn\(^{2+}\) permeation was observed in PC-3 cells, using this paradigm (see Figure 1A), indicating that phosphorylation of ERK1/2 was trigged by extracellular Zn\(^{2+}\). We found that extracellular Zn\(^{2+}\) responses triggered by Zn\(^{2+}\) are shown. Subsequently, ATP (100 μM) and TG (1 μM) were added to induce store depletion and Zn\(^{2+}\) was then reapplied. No apparent Zn\(^{2+}\)-dependent response is shown after store depletion. We next addressed the role of the IP\(_3\) pathway and possible cross talk between the MAPK and PI3K pathways. The application of the PKC inhibitor, YM254890 (1 μM), exhibited very low Zn\(^{2+}\) permeation was observed in PC-3 cells, using this paradigm (see Figure 1A), indicating that phosphorylation of ERK1/2 was trigged by extracellular Zn\(^{2+}\). We found that extracellular Zn\(^{2+}\) triggered prolonged phosphorylation of both ERK1/2 and AKT, which lasted at least 3 h. Rates of Zn\(^{2+}\)-dependent activation of ERK1/2 and AKT were different as maximal phosphorylation of ERK1/2 was apparent immediately after extracellular Zn\(^{2+}\) application, whereas phosphorylation of AKT increased during the first 1.5 h period following application of Zn\(^{2+}\) (100 μM).

We next addressed the role of the IP\(_3\) pathway and possible cross talk between the MAPK and PI3K in mediating Zn\(^{2+}\)-dependent signaling. Zn\(^{2+}\)-dependent phosphorylation of ERK1/2 was completely blocked by the application of the MEK1 inhibitor, U0126 (1 μM), indicating that Zn\(^{2+}\) permeation was observed in PC-3 cells, using this paradigm (see Figure 1A), indicating that phosphorylation of ERK1/2 was trigged by extracellular Zn\(^{2+}\). We found that extracellular Zn\(^{2+}\) responses triggered by Zn\(^{2+}\) are shown. Subsequently, ATP (100 μM) and TG (1 μM) were added to induce store depletion and Zn\(^{2+}\) was then reapplied. No apparent Zn\(^{2+}\)-dependent response is shown after store depletion. We next addressed the role of the IP\(_3\) pathway and possible cross talk between the MAPK and PI3K pathways. The application of the PKC inhibitor, YM254890 (1 μM), exhibited very low Zn\(^{2+}\) permeation was observed in PC-3 cells, using this paradigm (see Figure 1A), indicating that phosphorylation of ERK1/2 was trigged by extracellular Zn\(^{2+}\). We found that extracellular Zn\(^{2+}\) triggered prolonged phosphorylation of both ERK1/2 and AKT, which lasted at least 3 h. Rates of Zn\(^{2+}\)-dependent activation of ERK1/2 and AKT were different as maximal phosphorylation of ERK1/2 was apparent immediately after extracellular Zn\(^{2+}\) application, whereas phosphorylation of AKT increased during the first 1.5 h period following application of Zn\(^{2+}\) (100 μM).

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exposure to Zn$^{2+}$ was shown previously to reduce prostate cell proliferation and induce cell death (32). Yet, prolonged exposure is expected to induce desensitization of intracellular signaling (Figure 3). We therefore employed a paradigm in which cells were only briefly exposed to Zn$^{2+}$. This paradigm simulates more accurately the physiological environment in which most of the extracellular Zn$^{2+}$ is bound, though it may produce a brief rise in free Zn$^{2+}$. To assess the effect of extracellular, Zn$^{2+}$-dependent signaling on cell growth, PC-3 cells were treated daily for 10 min with Zn$^{2+}$-containing (10 or 100 lM) Ringer's solution and cell number was monitored using sulforhodamine B stain. Zn$^{2+}$-dependent intracellular signaling was fully activated under these conditions (see insert in Figure 5A), yet desensitization or Zn$^{2+}$ permeation was not apparent. To eliminate residual Zn$^{2+}$, Ringer's solution containing the Zn$^{2+}$ chelator, CaEDTA (100 lM, 10 min), was used as an additional control. Cells were grown in serum-free medium to minimize the interference of Zn$^{2+}$ contamination or serum-derived growth factors. As shown in Figure 5A, the growth rate of cells treated with nominally Zn$^{2+}$-free solution (control) or CaEDTA was attenuated. The brief daily exposure to 100 lM Zn$^{2+}$ enhanced PC-3 proliferation, already on the third day of treatment, by ~30% compared with control cells (Figure 5A). By the fifth day, the number of Zn$^{2+}$-treated cells increased further and was 2-fold higher than the controls. The Zn$^{2+}$-dependent enhancement of cell growth was dose dependent, as application of 10 lM Zn$^{2+}$ induced only about a 35% increase in cells by the fifth day.

To determine the effect of desensitization on Zn$^{2+}$-dependent proliferation, PC-3 cells were treated daily with Zn$^{2+}$-Cit (using the same experimental paradigm described in Figure 3), followed by a short exposure to Zn$^{2+}$ (100 lM). Application of citrate alone (see Figure 3) served as a control. The number of control cells treated with citrate alone and then with Zn$^{2+}$ increased by 4-fold on the fifth day (Figure 5B). In contrast, pre-exposure of PC-3 cells to Zn$^{2+}$-Cit completely attenuated Zn$^{2+}$-dependent cell growth (Figure 5B). In fact, the number of cells pretreated with Zn$^{2+}$-Cit decreased by the fifth day, similar to the effect of CaEDTA. These results indicate that extracellular Zn$^{2+}$ promotes cell growth suppressed by Zn$^{2+}$-Cit.

We next asked whether extracellular Zn$^{2+}$ also has a prosurvival effect. PC-3 cells were treated daily for 4 days with Zn$^{2+}$; the Zn$^{2+}$...
chelator CaEDTA or Ringer’s solution (control) and subjected to trypan blue staining (Figure 5C). Brief daily exposure to Zn$^{2+}$ resulted in a significant reduction in the number of trypan blue stained (i.e. dead) cells. Zn$^{2+}$ chelation with CaEDTA resulted in a 10-fold increase in trypan blue staining compared with control cells. These results suggest that the effects of extracellular Zn$^{2+}$ on cell growth are also mediated by attenuation of cell death.

Zn$^{2+}$-dependent enhancement of cell growth is not observed in prostate cancer cells which are deficient in ZnR activity.

Several sublines of PC-3 cells, differing in their metastatic potential and their response to chemotherapeutic drugs, have been described and attributed to their relatively high genetic instability and phenotypic drift (33,34). We therefore screened PC-3 lines from several sources, aiming to identify one not exhibiting ZnR activity (33), data not shown. These results indicate that PC-3 and PC-3B sublines differ in their ZnR activity, providing a useful experimental tool to address the specific role of this receptor in prostate cancer cells.

To determine the specific role of ZnR signaling in linking changes in extracellular Zn$^{2+}$ and the activation of MAPK and PI3K pathways, we compared the effects of extracellular Zn$^{2+}$ on ERK1/2 and AKT phosphorylation in the ZnR-deficient subline, PC-3B. Zn$^{2+}$-dependent phosphorylation of ERK1/2 in the PC-3B subline was insignificant compared with control, untreated cells (Figure 6C) and much smaller than the Zn$^{2+}$-dependent increase in ERK1/2 phosphorylation in PC-3 cells, indicating that the MAPK pathway is functional in these cells. Basal phosphorylation of AKT in PC-3B cells, in the absence of Zn$^{2+}$, was higher than in the PC-3 cells (Figure 6C, note the left lane of the immunoblot as well as the scale of the densitometry as compared with Figure 4B). No significant Zn$^{2+}$-dependent phosphorylation of AKT was apparent, whereas ATP (100 μM) induced a rise in AKT phosphorylation by ~7-fold, indicating the PI3K pathway is intact in the PC-3B cells. Thus, our results indicate that extracellular Zn$^{2+}$-dependent activation of the MAPK and PI3K pathways is observed only in PC-3 cells exhibiting Zn$^{2+}$-dependent metabotropic signaling.

Fig. 5. Extracellular Zn$^{2+}$-dependent cell growth is attenuated by ZnR desensitization. (A) PC-3 cells were treated daily for 10 min with Ringer’s solution containing 10 (triangles) or 100 μM Zn$^{2+}$ (circles); 100 μM CaEDTA (diamonds) or with Ringer’s alone (control, open squares). Cell number was monitored using a Coulter counter. The respective Zn$^{2+}$-dependent Ca$^{2+}$ response of the cells is shown in the inset. (B) PC-3 cells were pretreated, daily, with Zn$^{2+}$ Cit (6 mM Zn$^{2+}$, 10 mM citrate) to desensitize ZnR (see Figure 3). Control cells were pretreated with citrate alone as marked. Then Zn$^{2+}$ (open circles and filled black squares), or CaEDTA (filled circles), was applied, daily, and cell number monitored as in A. Following Zn$^{2+}$ Cit pretreatment, at concentration which induces ZnR desensitization, the Zn$^{2+}$-dependent cell growth was abolished. Note that following Zn$^{2+}$ Cit pretreatment the growth curves in the presence of Zn$^{2+}$ or CaEDTA (black squares or filled circles) are overlapping. (C) PC-3 cells, treated as in A, were stained using trypan blue on the fourth day. Averaged number of trypan blue-stained cells in the field of view (×10), in each treatment, is shown together with a representative light microscope image.
this cancer are urgently sought. Among the most intriguing but less understood changes occurring in prostate cancer transformation is a 10-fold decrease in the concentration of Zn$^{2+}$. In recent years, this ion has emerged as an important signaling agent, regulating downstream pathways involved in cell proliferation and survival (14,36). However, the signaling pathways activated by extracellular Zn$^{2+}$ in healthy and malignant prostate tissue remain unknown.

In the present work, we identified extracellular, Zn$^{2+}$-dependent metabotropic Ca$^{2+}$ release in androgen-independent PC-3 and DU-145 prostate cancer cell lines and in the mouse prostate tumor TRAMP-1 cell line, but not in the androgen-sensitive LNCaP cells. Interestingly, the androgen-insensitive PC-3 cells exhibited two sublines that showed distinct ZnR activity. Our results, therefore, do not support a clear link between androgen sensitivity and extracellular Zn$^{2+}$ response. Previous work, however, has suggested that changes in androgen sensitivity of LNCaP cells may be followed by changes in intracellular Zn$^{2+}$, Zn$^{2+}$-buffering proteins, such as Zn$^{2+}$ transporters and metallothioneins, as well as cell growth (37,38). This is consistent with other reports of decreased Zn$^{2+}$ permeability in androgen-insensitive cells and malignant tissue, linked to a reduced expression of ZIP1 (12,39). Additional studies are needed to determine if a link exists between Zn$^{2+}$ signaling, intracellular Zn$^{2+}$ concentration and androgen sensitivity.

In the present report, we have shown that extracellular Zn$^{2+}$ triggers the IP$_3$ pathway in PC-3 cells, leading to release of intracellular Ca$^{2+}$. This Ca$^{2+}$ rise is mediated by a Gq-protein and activation of PLC and is abolished following depletion of TG-sensitive Ca$^{2+}$ stores. This subsequently leads to activation of MAPK and PI3K. This signaling pathway was previously attributed to a GPCR, termed ZnR (14,22,23). Our data further indicate that a brief rise in extracellular Zn$^{2+}$ plays a role in promoting prostate cancer cell growth only in those cells exhibiting ZnR activity (i.e., PC-3 but not PC-3B cells). The results presented here indicate that prostate cancer cells are largely impermeable to Zn$^{2+}$, in agreement with previous studies (2,6,12,13). Although the apparent K$_{0.5}$ of the prostate ZnR is 200 µM, our results indicate that Zn$^{2+}$-dependent signaling in prostate cancer cells is activated, and induces enhanced proliferation, already at 10 µM. It is further important to note that even in the presence of citrate, Zn$^{2+}$ can trigger a ZnR response at slightly higher free Zn$^{2+}$ concentrations. Interestingly, Zn$^{2+}$Cit complex led to functional desensitization of ZnR-mediated, intracellular Ca$^{2+}$ rise. Furthermore, Zn$^{2+}$-induced desensitization was followed by attenuation of Zn$^{2+}$-dependent cell growth. The concentrations of both Zn$^{2+}$ and citrate used in our experiments are similar to those found in the non-neoplastic prostate, suggesting that ZnR is quiescent in the prostate tissue. One of the hallmarks of prostate cancer progression is a dramatic decrease in Zn$^{2+}$ and citrate concentrations that lower the buffer capacity for free Zn$^{2+}$ in the prostate (40). Furthermore, because of the relatively low affinity of Zn$^{2+}$ to citrate, even a modest change in citrate concentration may dramatically alter the concentration of free Zn$^{2+}$, thereby triggering a transient rise in Zn$^{2+}$ and activation of the Ca$^{2+}$ response. This suggests that a scenario in which ZnR is transiently activated in the prostate by a rise in extracellular Zn$^{2+}$ may also be considered. This further suggests that desensitization by Zn$^{2+}$Cit plays an important role in suppressing tumor growth. The role of ZnR signaling would be expected to be more pronounced following metastasis of prostate tumor cells into peripheral tissues in which the extracellular Zn$^{2+}$Cit concentration is negligible, minimizing ZnR desensitization. Several plausible mechanisms may trigger a transient rise in Zn$^{2+}$ concentration in the peripheral tissues. Among these, is the tissue destruction mediated by tumor invasion which can lead to release of Zn$^{2+}$ from injured cells. In addition, NO and oxidative signaling mediated by the tumor cells may induce release of Zn$^{2+}$ from metalloproteins (41). Finally, in bone, arguably the most common and devastating target of prostate metastatic cells, the high concentrations of Zn$^{2+}$ released during bone destruction occurring in metastatic prostate cancer could potentially activate the ZnR (42). Consistent with this hypothesis is the finding that ZnR activity was particularly intense in the metastatic prostate cancer cell lines.

**Discussion**

Prostate cancer is the second most common malignancy in men in Western countries and a major cause of death. While in the early stage, prostate cancer is treatable by hormones, the advanced stage is androgen insensitive and an effective therapeutic approach is still unavailable. Thus, novel molecular targets to facilitate treatment for
Therefore, extracellular Zn\textsuperscript{2+} signaling in prostate cancer represents an important pathway linking changes in Zn\textsuperscript{2+} to prostate tumor cell growth and survival.

The identification of a PC-3 subline, PC-3B, that is deficient in extracellular Zn\textsuperscript{2+}-dependent signaling, provides an important tool to assess the role of the Zn\textsuperscript{2+}-dependent metabolic response in prostate cancer. The spontaneous formation of PC-3 subline with distinct morphological and functional characteristics is common and is attributed to their genetic instability (33,34). It would be of interest to further characterize these sublines, for example, to determine if the difference in the response to extracellular Zn\textsuperscript{2+} is also accompanied by a change in the expression pattern of other zinc transporters involved in prostate cancer (5,12).

The Zn\textsuperscript{2+}/Cit complex, at concentrations that did not trigger ZnR activity, induced a profound functional desensitization of the Zn\textsuperscript{2+}-dependent activity. Desensitization by Zn\textsuperscript{2+}/Cit was similar to the desensitization induced by Zn\textsuperscript{2+} alone, suggesting that the Zn\textsuperscript{2+}/Cit complex may allosterically interact with the ZnR. Although the molecular basis for this effect is still unknown, it is reminiscent of the constitutive desensitization encountered in other GPCRs (43). For example, such desensitization, without prior activation of the intracellular signaling, has been attributed to activation of GPCR kinases. Constitutive desensitization has also been described for the serotonin receptor, 5-hydroxytryptamine (44), a member of the metabotropic glutamate receptor family.

The MAPK- and PI3K-signaling pathways participate in a wide variety of physiological processes and have been shown to induce proliferation of prostate cancer cells (29,45). The PI3K pathway is prominent in prostate cancer, where constitutively activated PI3K has been correlated with tumor cell survival (46). Activation of PI3K has also been suggested to downregulate the androgen receptor, thereby inducing the shift toward advanced stages of this cancer (47). Furthermore, PI3K activation was reported to induce chemoresistance of prostate cancer cells (48). We have shown that the Zn\textsuperscript{2+}-dependent MAPK and PI3K activation is correlated with enhancement of cell growth in PC-3 cells which exhibit ZnR activity. Our results indicate that extracellular Zn\textsuperscript{2+} is inducing the activation of MAPK at least partially via a signaling pathway involving PI3 and PKC. A derivative of prostaglandin also activated this signaling pathway to trigger MAPK activation in PC-3 cells (49). It was recently shown that MAPK activation is triggered by Zn\textsuperscript{2+} in prostate cancer cells (13); this effect, however, was produced only after addition of a Zn\textsuperscript{2+}-ionophore. It is possible that in the presence of the ionophore, the activation of MAPK was also mediated via a distinct pathway. The ERK pathway has been linked to both proliferation and to attenuation of cell growth. While it was previously suggested that the rise in intracellular Zn\textsuperscript{2+}, in the presence of the Zn\textsuperscript{2+} ionophore, inhibited nuclear factor-kappa B activity and suppressed the invasiveness of PC-3 cells (13), our work indicates that extracellular Zn\textsuperscript{2+} enhanced cell growth. These distinct effects of extracellular or intracellular Zn\textsuperscript{2+} on cell fate are consistent with previous studies (36,50,51). Activation of AKT by extracellular Zn\textsuperscript{2+} via the ZnR was largely mediated by the IP\textsubscript{3}-dependent pathway, as the Gq and PKC inhibitors attenuated AKT phosphorylation. Previous work has suggested that the Ca\textsuperscript{2+}-independent PKC\textsubscript{c} is a major inhibitor of AKT which leads to apoptosis (52). Our results, therefore, indicate that another isoenzyme of PKC is activated by extracellular Zn\textsuperscript{2+} leading to cell survival. In support of this conclusion, the Ca\textsuperscript{2+}-dependent PKC\textsubscript{c} was shown previously to activate ERK/1/2 via transactivation by epidermal growth factor receptor in PC-3 cells (53). The prolonged 3 h period that the phosphorylation of AKT persists following a short exposure to Zn\textsuperscript{2+} underscores the role of Zn\textsuperscript{2+} in activating signaling, leading to enhanced tumor cell growth. Finally, attenuation of the Zn\textsuperscript{2+}-dependent phosphorylation of ERK/1/2 by a PI3K inhibitor, and a similar effect of a MEK inhibitor on PI3K activation, indicate that these pathways interact to promote Zn\textsuperscript{2+}-dependent cell proliferation, similar to the effect previously observed in Zn\textsuperscript{2+}-dependent signaling in colon cells (14,54). Taken together, our results indicate that Zn\textsuperscript{2+} activates several signaling pathways that interact to enhance tumor progression. The lack of a Zn\textsuperscript{2+}-dependent phosphorylation of the MAPK and PI3K in ZnR-deficient, PC-3B cells, indicates that activation of both pathways is primarily mediated by the ZnR.

In conclusion, our results show that extracellular Zn\textsuperscript{2+} acts as a signaling agent via the ZnR in prostate cancer cells. Our results further identify a unique role for Zn\textsuperscript{2+}/Cit as an effective suppressor of extracellular Zn\textsuperscript{2+} signaling. The finding that a rise in extracellular Zn\textsuperscript{2+} significantly enhances prostate cancer cell proliferation and survival suggests that blocking extracellular Zn\textsuperscript{2+} signaling can be a safe and effective therapeutic strategy to attenuate prostate cancer cell growth and tumor progression.

**Supplementary material**

Supplementary Figure 1 can be found at http://carcin.oxfordjournals.org/

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