

Involvement of Ion Channels in Endothelin-1-induced Signalling in Human Prostate Cancer Cells

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Abstract

Objective: Endothelin-1 (ET-1), a potent vasoconstrictor secreted primarily by endothelial and various epithelial cancer cells has been implicated in prostate cancer progression and the ET axis has been suggested to represent a novel and exciting target in the treatment of prostate cancer (PCa). ET-1, acting primarily through the endothelin receptors (ETRs) is integrally involved in multiple facets of PCa progression, including cell growth, inhibition of apoptosis, angiogenesis and development of bone metastases. ET-1 and ETRs are expressed in PCa tissues and their expression is modulated during the evolution of these cancers. The purpose of the present work was to study the effects of ET-1 on proliferation of human PCa cells PC-3 and the mechanisms by which the activation of ETRs may promote the PCa cells growth.

Methods: Prostate cancer cell lines and primary cultured epithelial cells from prostate cancer, RT-PCR and calcium imaging techniques were used to study the expression and functionality of the Endothelin receptors and involvement of ion channels in the effects of ET-1 in prostate cancer cells.

Results: We show for the first time that the application of ET-1 induces a dose-dependent cell proliferation and an increase in intracellular free Ca^{2+} concentrations ($[Ca^{2+}]_i$) via a mobilisation of the internal calcium stores and by a capacitative calcium entry (CCE). These effects of ET-1 were completely abolished by BQ123, a selective ETAR antagonist, but not by BQ788, a selective ETBR antagonist. By use of pharmacological inhibitors and siRNA targeting calcium-activated (IKCa1 and BKCa) potassium channels and calcium channels (TRPC1, TRPV6, Orai1), we showed that these ion channels play an important role in calcium entry and cell proliferation induced by ET-1 in PCa cells.

Conclusion: These results suggest that these ion channels evidenced here might constitute potential targets to block the ET axis in human prostate cancers.

Keywords: Endothelin-1; Calcium signalling; Calcium-activated Potassium channels; Capacitative calcium entry; Orai-1; TRPV6; TRPC1 ion channels; Prostate cancer; Proliferation

Abbreviations: ETs: Endothelins; ET-1: Endothelin-1; ETRs: Endothelin Receptors; ETAR: Endothelin A Receptor; ETBR: Endothelin B Receptor; PCa: Prostate Cancer; IKCa1: Ca^{2+} -activated K^+ Channel with Intermediate Conductance; BKCa: Ca^{2+} -activated K^+ channel with big conductance; TRP channels, RT-PCR: Reverse Transcription-polymerase Chain Reaction; CRAC: Calcium Release-activated Ca^{2+} Channel; CCE: Capacitative Calcium Entry; SOCE: Store Operated Calcium Entry.

Introduction

Prostate cancer is characterized by low rates of cell proliferation coupled with diminished rates of cell death [1]. This pattern has made prostate cancer among the most resistant of malignancies to cytotoxic chemotherapeutic agents. Furthermore, the cornerstone of the management of advanced prostate cancer, androgen deprivation therapy, relies on the effective induction of apoptotic pathways. The emergence of androgen refractory prostate cancer, which leads to the lethal form of the disease, indicates that these cells likely have developed survival mechanisms to escape death.

Recent studies in the understanding of prostate cancer biology have led to the development of drugs directed against precise molecular alterations in the prostate tumor cells. Endothelins (ETs) and their receptors have emerged as a potential target in prostate cancer. The potent vasoconstrictor endothelin-1 (ET-1) has been implicated in prostate cancer disease progression [2-4]. ET-1 expression occurs in almost every human prostate cancer tissue studied [4,5] and the expression increases in metastatic PCa [2,3]. Interest in the role of ET-1

in cancer has grown over the last decade since studies [6], Shichiri et al. [7] reported that several human tumor cell lines released ET-1 into the culture medium and that this stimulated proliferation of these cells. By activating of the Endothelin receptors (ETRs), ET-1 is involved in several aspects of prostate cancer progression including proliferation, escape from apoptosis, invasion, and new bone formation, processes that are general to many malignancies. Moreover, patients with metastatic prostate cancer have elevated levels of plasma ET-1 compared with patients with organ-confined cancer as well as healthy individuals [4]. Biological activity of the ETs is mediated by the activation of two G protein-coupled receptor subtypes, endothelin receptor A (ETAR) and B (ETBR) interacting with heterotrimeric G proteins. Each G-protein activates several downstream effectors [8] through the activation of phospholipase C (PLC) [9], which cleaves phosphatidylinositol bisphosphates generating two second messengers IP3 and DAG leading to an increase in intracellular calcium levels and an activation of protein kinase C (PKC) and MAPK. These transduction pathways might relay the mitogenic signal induced by ETs to the nucleus and promote cell

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proliferation. Activation of the endothelin receptor A (ETA) can lead to induction of a survival pathway, whereas activation of the endothelin receptor B (ETB) can result in clearance of circulating ET-1 as well as in stimulation of apoptosis. However, the response to the binding of either receptor remains cell type-dependent. In prostate cancer, the expression of the endothelin receptors, ETAR and ETBR, is altered compared to the pattern seen in normal prostatic tissues [5,10]. The ETB, predominant on benign prostatic epithelial cells, has a much lower expression on prostate cancer cells, owing, at least in part, to frequent hypermethylation of the ETB gene, *EDNRB* [11]. Increased ET-1 expression, coupled with the increased ETA expression that occurs with higher prostate tumor stage and grade, may produce a survival advantage for the prostate cancer cells. Specific ETAR antagonists may therefore provide an improved approach in tumor treatment in which ETAR blockade could result in tumor growth inhibition. Selective ETAR antagonists may block the proliferative effects of exogenous ET-1 in both prostate cancer cells and osteoblasts by direct effects on the tumor cells. Indeed, in a phase III clinical trial of the ETA antagonist, atrasentan, there was a significant delay in time to disease progression compared to placebo in men with hormone refractory disease [12,13]. Clinical trials of atrasentan has also demonstrated benefits in prostate cancer progression [14] and this drug is actually in phase 3 of clinical trial [15] decreased pain in men with prostate cancer.

Studies have shown that the ET-1-induced elevation of intracellular free Ca^{2+} ($[Ca^{2+}]_i$) in different cell systems. An elevation of $[Ca^{2+}]_i$ is known to play an important role in cell growth, survival, migration and apoptosis. According to the published data, the elevation of $[Ca^{2+}]_i$ caused by ET-1 is controlled by different pathways including a transmembrane Ca^{2+} influx and an intracellular Ca^{2+} release via the activation of inositol trisphosphate receptor (IP3R) and of ryanodine receptors (RyRs). Increasing number of works show the implication of potassium and calcium channels in the ET-1 effect on the cell proliferation [16]. Early evidence for the role of the $[Ca^{2+}]_i$ in ET-1 induced proliferation of tumor cells was described by Shichiri et al. [7] in epithelial carcinoma cell lines derived from the human cervix (HeLa) and larynx (Hep-2). They found that ET-1 stimulated both Ca^{2+} signalling and proliferation of quiescent cells in a dose-dependent manner. Similar results were reported in ovarian cancer cells by Bagnato et al. [17]. Although the presence of ET-1 and receptors mRNA and proteins have been shown in human prostate cancer tissues and

prostate cancer cell lines [18], actually no information is available about the ET-1 and calcium signalling in prostate cancer.

In a previous work, we have shown that the calcium-activated IKCa1 potassium channel controls prostate cancer cell proliferation by a close regulation of passive calcium entry via TRPV6 [19]. Even though little is known about the effects of ET-1 on the calcium signalling in PCa cells, the involvement of ion channels in ET-induced calcium signalling and the involvement of ion channels in ET-1-induced cell proliferation. In the present study, we show for the first time the mitogenic effect of ET-1 and ETRs in human prostate cancer cells PC-3 and primary cultures human prostate cancer cells (PrEC) and then, we explored the effects of ET-1 on $[Ca^{2+}]_i$ and determined the involvement of the Ca^{2+} -activated K⁺ channels and of the plasma membrane calcium channels in ET-induced Ca^{2+} signalling. We further studied the ET-1 the involvement of the ion channels in ET-1-induced cell proliferation in human prostate cancer cells. The data presented here suggest that the ion channels constitute interesting targets in therapies to disrupt the ET-1 axis in human prostate cancer.

Materials and Methods

Reagents and chemicals

All chemicals were from Sigma (l'Isle d'Abeau, France), except for fura-2/AM purchased from France Biochem (Meudon, France).

Cell culture

LNCaP, DU45 and PC-3 prostate cancer cell lines, obtained from the American Type Culture Collection (ATCC), were cultured in RPMI supplemented by 10% fetal calf serum (FCS) as previously described by [20]. Primary cultured prostate cancer epithelial cells (PrCE) were obtained and cultured as described in a previous work [21]. Before experiments, cells were cultured in 2% FCS for 48 hours.

RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated as described by Chomczynski et al. [22]. After DNase I (Invitrogen) treatment to eliminate genomic DNA, RT-PCR experiments were carried out using a GeneAmp PCR system 2400 thermal cycler (Applied Biosystems), as previously described [23]. The PCR primers used in this study (Table 1) were designed on the basis of established

Gene	5'-forward-3'	5'-reverse-3'	RT-PCR product Size (bp)	Accession number
ET _A R	GATCACAATGACTTTGGCGTA	CTCAAGCTGCCATTCTTCT	328	NM_001957
ET _B R	TTGGTGGCTGTTGAGTTCT	TGCTCACCAGATACAGAGCA	351	NM_000115
IK _{Ca1}	ACTGGTTCGTGGCCAGCTTTACA	TCCAGCAGCCATCAGCAAGCCA	867	NM_002250
BK _{Ca}	AGTACAAGTCTGCCAACCAGAGAGA	TCAAAGCCGCTCTTCTGCACGTAC	1634	NM_002247
SK3	TCATGAAGACGCTCATGACCA	TGGAGGAATCTCTCTGGTGT	469	NM_002249
TRPC1	AGCGCATGTGGCAATCTTTGT	CGCTCCATGATTAGAAAATGG	595	NM_003304
TRPV6	ATGGTGATGCGGCTCATCAGTG	GTAAGAGTGGCTAGCTCCTCG	255	XM_001126541
Orai1	CTTCTTCGACCTCGTCTCCT	CGTAAGGCCAAGCATGGAA	406	NM_032790
STIM1	CCAACAGTGAACACAGCAC	CATCATCTTCTCATGTGCTCC	435	JX014264
b-actin	CAGAGCAAGAGAGGCATCCT	GTTGAAGGTCTCAAACATGATC	212	NM_001101
siRNA	Target sequences (5'-3')			
TRPC1	GGGUGACUUAUUAUGGUU			NM_001251845
TRPV6	GACUCUCUAUGACCUCACA			NM_018646
Orai1	UGAGCAACGUCACAAUCU			NM_032790.3
IK _{Ca1}	UGUAAAGCUUGGCCACGAAC			NM_002250
BK _{Ca}	GAGUCCUGGUUGUCUAGU			XM_017016207

Table 1: RT-PCR Oligonucleotides and siRNA target sequences.

GenBank sequences and synthesized by Invitrogen. The expression of β -actin mRNA was used as an internal standard. PCRs were carried out using 1 μ l of the RT reaction the total RNA extracted from cells. The conditions were as follows: 7 min at 95°C, then 40 cycles of 30 sec extension at 72°C each, 30 sec to 1 minute according to the size of amplicons and a final 7 min extension step. Half of the PCR samples were analyzed by electrophoresis in a 1.5% agarose gel, stained with ethidium bromide (0.5 μ g/ml) and viewed by Gel Doc 1000 (Bio-Rad, Hercules, CA, USA).

siRNA cell transfection

PC-3 cells were transfected overnight with 20 nM siRNA control (siRNA number 1, Dharmacon Inc.) or raised against IKCa1, BKCa, Orai-1, TRPV6 and TRPC1 (Table 1) using GenePorter transfection reagent (Gene Therapy Systems, Inc., GTS, San Diego) in serum-free DMEM, according to the manufacturer's recommended protocol. The cells were then incubated in RPMI medium containing 10% SVF for two days to down-regulate the expression of these targets (mRNA) prior to use in experiments.

Cell growth assays

Cell growth assays were performed as previously described by [24]. Cells were incubated for 4 days in a medium containing the solvent (DMSO) either alone (control) or with ET-1, U73122, U73433 at various concentrations. Treatments by the siRNA targeting ions channels mRNA was performed as described above. The selective ETAR antagonist BQ123 [25] and the selective ETBR antagonist BQ788 [26] were used. Since previous studies described ET-1 as a co-mitogen requiring low concentrations of serum for maximal mitogenic activity [27], assays for ET-1-induced mitogenic effects in this study were performed by incubating the PC-3 cells in culture medium containing 2% SVF. The commercial assay kit, consisting of MTS (inner salt) and PMS (an electron coupling reagent), was then used to estimate cell growth. One-hundred percent reflected the number of viable cells after incubation in control medium without drugs for 4 days.

Measurement of $[Ca^{2+}]_i$

PC-3 and LNCaP cells were grown on glass coverslips for Ca^{2+} imaging experiments. $(Ca^{2+})_i$ was measured using fura-2-loaded cells, as previously described [19]. Briefly, cells were loaded with 2 μ M fura-2/AM (fura-2 acetoxymethyl ester) (Calbiochem, Meudon, France) in HBSS solution (Hank's Balanced Salt solution) containing (in mM): 142 NaCl, 5.6 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 0.34 Na_2HPO_4 , 0.44 KH_2PO_4 , 4.2 $NaHCO_3$, 10 HEPES, and 5.6 glucose, at room temperature for 45 min. All recordings were carried out at room temperature. The cells were continuously perfused with the HBSS solution and chemicals were added via the perfusion system. The flow rate of the whole-chamber perfusion system was set to 1ml/min and chamber volume was 500 μ l.

Statistical analysis

Plots were produced using Origin 5.0 (Microcal Software, Inc., Northampton, MA). Results are expressed as mean $\pm$ S.E. Statistical analysis was performed using unpaired t tests or ANOVA tests followed by either Dunnett (for multiple control versus test comparisons) or Student-Newman-Keuls post-tests (for multiple comparisons). Student's t-test was used for statistical comparison of the differences, and $P < 0.05$ was considered significant.

Results

Expression of ETAR and ETBR in human prostate cancer cells

The RT-PCR technique was used to study the mRNA expression

of ETAR and ETBR in human PCa cell lines (LNCaP, PC-3) and primary cultured human prostate cancer epithelial (PrEC) cells from tissue specimens. The specific primers for ETAR and ETBR allowed to amplify the PCR products of the expected sizes for ETAR (328 bp) and ETBR (351 bp) from the cDNA of all cell lines and primary cultured epithelial cells (PrEC) (Figure 1A). The identity of the amplicons was verified by cloning and sequencing and corresponded to the cDNA of the human ortholog of these receptors.

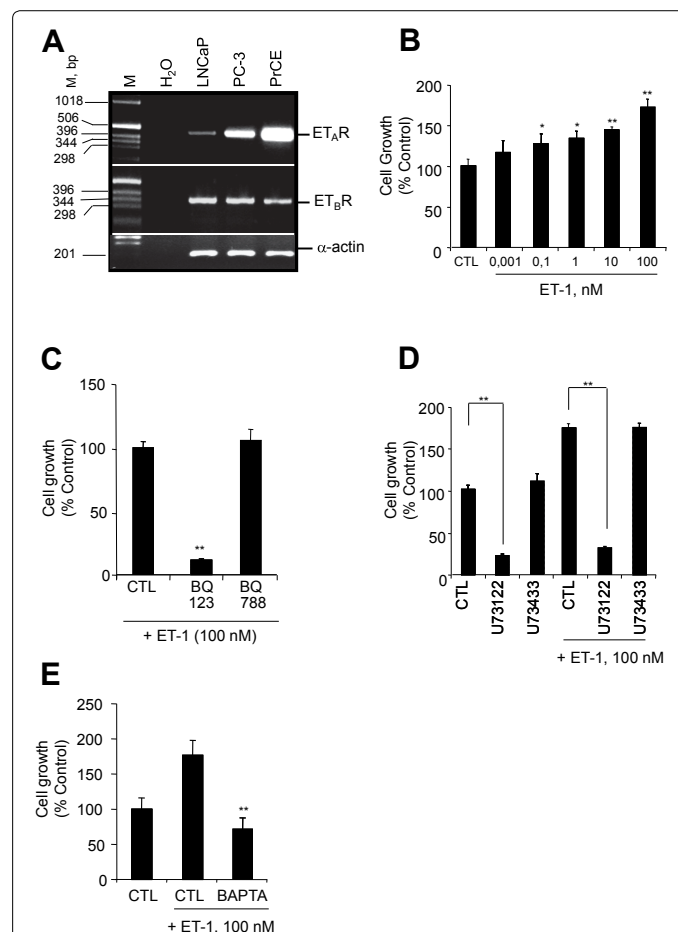


Figure 1: The effects of ET-1 on the cell proliferation in prostate cancer cells. (A) RT-PCR analysis of ETAR and ETBR expression in androgen-sensitive (LNCaP), androgen-insensitive (PC-3), and primary cultured human prostate cancer epithelial (PrEC) cells. The amplified cDNA fragments with respective specific primers are 328-bp for ETAR and 351-bp for ETBR. (B) ET-1 stimulated a dose-dependent cell growth in PC-3 cells. Cells in 96-well plates were rendered quiescent in RPMI 1640 medium with 2% FCS. Quiescent monolayer cells were stimulated with ET-1 at the indicated concentrations and then cultured for a further 4 days. (C) ET-1-induced cell proliferation was blocked by ETAR antagonist BQ123. ET-1 (100 nM) was added to quiescent cells or to quiescent cells pre-incubated for 10 min at 37°C with BQ123 (1 μ M) or BQ788 (1 μ M) then cultured for a further 4 days before MTS assay. (D) ET-1-induced cell proliferation was significantly reduced by U73122 (5 μ M), a specific inhibitor of phospholipase C (PLC) then cultured for a further 4 days before MTS assay. ET-1 (100 nM) was added to quiescent cells or to quiescent cells preincubated for 10 min at 37°C with U73122 or with the inactive U73122 analogue U73433. (E) ET-1-induced cell proliferation was blocked by pre-incubation of the cells with BAPTA/AM (10 μ M), a membrane permeant form of BAPTA, a calcium-chelating molecule. RT-PCR experiments were confirmed in at least 4 different cell cultures and a representative figure is presented. Cell growth studies were performed in 96-well plates (8 wells/condition) and results are shown as the mean $\pm$ SE. Experiments were repeated four times in independent cell cultures and a representative figure is presented for each cell type. Results are presented as the percentage of corresponding controls (CTL). * $P < 0.05$; ** $P < 0.001$ compared with their respective controls.

Effects of ET-1 on human prostate cancer cells proliferation

The effects of ET-1 were studied on the proliferation rates of prostate cancer cell line PC-3 and PrEC. After 4 days of incubation, ET-1 induced significant and dose-dependent increase in cell proliferation at the concentrations tested (0,001-100 nM) reaching the maximal effect at 100 nM for PC-3 cells (Figure 1B) cells. To define the ET-1 receptors involved in the action of ET-1 on the proliferation of PC-3 cells, highly selective ETAR and ETBR antagonists were used. In these experiments, the ET-1 (100 nM)-induced cell proliferation was abolished by BQ123 (1 μ M), a highly selective ETAR antagonist, but not by BQ788 (1 μ M), a highly selective ETBR antagonist (Figure 1C).

In the same manner, we also studied the involvement of a phospholipase C (PLC) in the ET-1 induced cell proliferation in PC-3 cells. As shown on Figure 1D, we observed that ET-1 (100 nM)-induced cell proliferation was reduced by 75% in the presence of the specific PLC inhibitor U73122 used at 10 μ M, but not by its inactive analogue U73433 (10 μ M). However, the inhibition of the proliferation induced by U73122 is not completed suggesting the involvement of other transduction mechanisms like as CD-38 pathway as described in smooth muscle cells [28]. Taking together, these experiments showed that the ET-1 induces cell proliferation in human PCa cells by activating the ETAR ET-1 receptor involving the PLC activation (ETAR /PLC pathway).

The role of $[Ca^{2+}]_i$ in ET-1-induced Cell proliferation

Responses regulated by ET-1 have been associated with increases in $[Ca^{2+}]_i$, either by influx of calcium or release of intracellular calcium stores (for review, see [29]). In order to study the involvement calcium in the ET-1-induced cell proliferation, the cells are treated with BAPTA to chelate the intracellular free calcium. For this, the cells were incubated with BAPTA/AcetoMethyl ester (BAPTA/AM) 30 min before the addition of ET-1 (100 nM). The proliferation rates were estimated 4 days after the treatments.

As shown in Figure 1E, in these experiments, the ET-1-induced cell proliferation was inhibited in the presence of the calcium chelator suggesting the involvement of the intracellular free calcium in the effects of ET-1 in PC-3 cell line. These data also suggest that ET-1 induces calcium signaling in the human PCa cells. These results indicated also that the increase in free cytosolic calcium concentration is implicated in the mitogenic effect of ET-1 on PC-3 cells.

We therefore studied the effects of ET-1 on free $[Ca^{2+}]_i$ in PC-3 cell line and in PrEC by calcium imaging using Fura-2-as a calcium probe. As shown in Figure 2, ET-1 (100 nM) induced the $[Ca^{2+}]_i$ response in PC-3 (Figure 2A) and in PrEC (Figure 2B).

Origin of ET-1-induced calcium response in prostate cancer cells

To explore the origin of the calcium response induced by ET-1 in prostate cancer cells, following experiments were performed. As illustrated in Figures 2C and 2D, application of ET-1 (100 nM) in calcium deprived medium (0Ca²⁺) induced a calcium rise originating from the release from internal stores and the subsequent capacitative calcium entry (CCE) in the same medium containing 2 mM of calcium (2Ca²⁺) in both PC3 cells (Figure 2C) and in PrEC (Figure 2D). In these experiments, the ET-1-induced increase in $[Ca^{2+}]_i$ was blocked by ETAR-selective receptor antagonist BQ123 (1 μ M), but not by the ETBR-selective receptor antagonist BQ788 (1 μ M) (Figure 2E). These data indicate that the ETAR expressed in the human prostate cancer

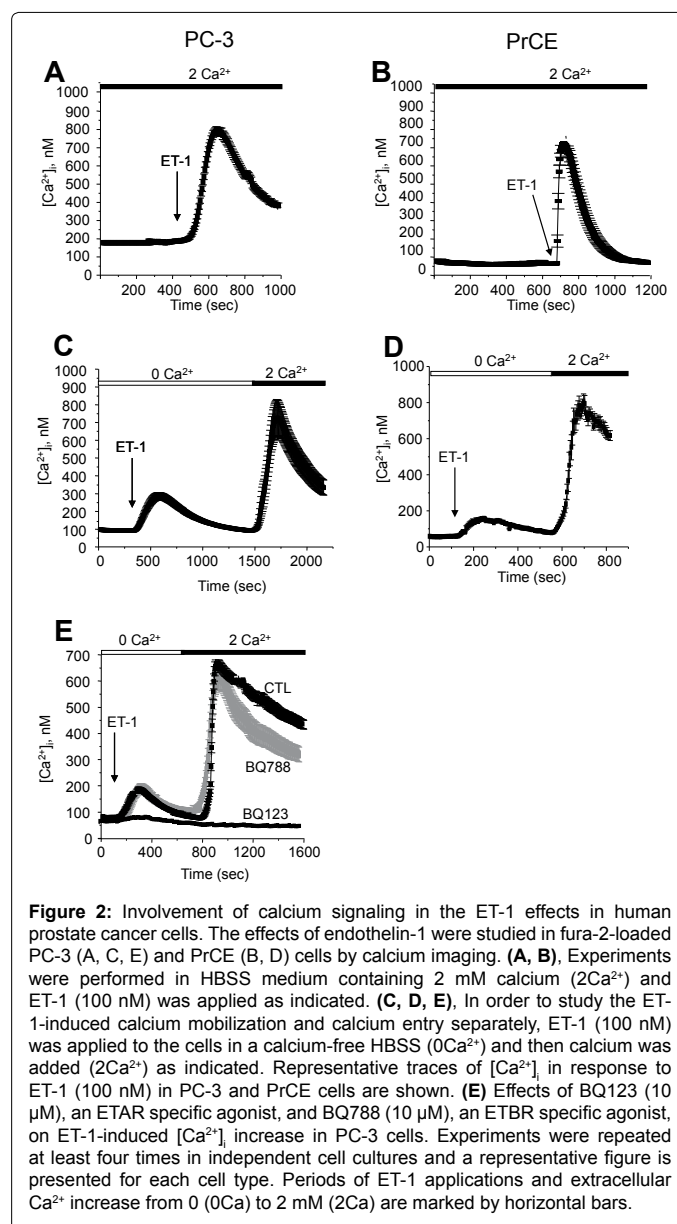


Figure 2: Involvement of calcium signaling in the ET-1 effects in human prostate cancer cells. The effects of endothelin-1 were studied in fura-2-loaded PC-3 (A, C, E) and PrEC (B, D) cells by calcium imaging. (A, B), Experiments were performed in HBSS medium containing 2 mM calcium (2Ca²⁺) and ET-1 (100 nM) was applied as indicated. (C, D, E), In order to study the ET-1-induced calcium mobilization and calcium entry separately, ET-1 (100 nM) was applied to the cells in a calcium-free HBSS (0Ca²⁺) and then calcium was added (2Ca²⁺) as indicated. Representative traces of $[Ca^{2+}]_i$ in response to ET-1 (100 nM) in PC-3 and PrEC cells are shown. (E) Effects of BQ123 (10 μ M), an ETAR specific agonist, and BQ788 (10 μ M), an ETBR specific agonist, on ET-1-induced $[Ca^{2+}]_i$ increase in PC-3 cells. Experiments were repeated at least four times in independent cell cultures and a representative figure is presented for each cell type. Periods of ET-1 applications and extracellular Ca²⁺ increase from 0 (0Ca) to 2 mM (2Ca) are marked by horizontal bars.

cells are functional and are linked to the intracellular Ca²⁺ signaling pathway, probably through the activation of the phosphoinositol/Ca²⁺ pathway. According to these data, the main pathway involved in the ET-1-induced calcium increase in PCa cells is the calcium entry following the opening of the membrane channels. We thus further investigated the ion channels involved in the calcium entry induced by ET-1 in PC-3 PCa cells.

Determination of the ion channels involved in the ET-1-induced CCE

Calcium entry involves the functionality of calcium channels and the potassium channels modulating the membrane potential and thereby the driving force for the calcium entry through the Voltage-independent calcium permeable channels. In a previous work [19], we showed that the Ca²⁺-activated K⁺ channels (IKCa1) are involved in the thapsigargin-induced CCE in prostate cancer LNCaP and PC-3 cells. In the present work, we studied the involvement of calcium-activated

K⁺ channels in the CCE-induced by ET-1. In the same manner, in our previous studies, we showed that TRPC1, TRPV6 and the Orai1/STIM1 complex were involved in the thapsigargin-induced CCE in prostate cancer cells [19,30-32].

First by using RT-PCR technique, we confirmed the expression of the calcium-activated potassium (IKCa1, BKCa and SK3) and calcium channels and their partners (TRPC1, TRPV6, Orai1 and STIM1) in prostate cancer cell lines (LNCaP, PC-3) and in PrEC cells from tissue specimens. Analysis of RT-PCR products revealed the amplification of a single fragment of the expected size for IKCa1 (867 pb) and BKCa (1600 bp), SK3 (440 bp), TRPC1 (595 bp), TRPV6 (255 bp), Orai1 (406 bp) and STIM1 (435 bp) from the cDNA of all cell lines and hPCE cells (Figures 3A and 3B). In order to assess the involvement of these ion channels in the CCE induced by ET-1 (100 nM), we used either the siRNA technology to down-regulate IKCa1 and BKCa or the pharmacological tools to inhibit the IKCa1 (TRAM-34, 10 μ M), the BKCa (Paxilline, 10 μ M) potassium channels or the calcium channels involved in SOCE (SKF96365, 10 μ M) in PC-3 cells.

The down-regulation of the ion channels mRNA and proteins are verified by RT-PCR and western blotting following the treatments of these cells by the specific siRNA used in the present study (data not shown). The cells were treated with ion channels targeting siRNA (20 nM) for 48 h and then, calcium imaging experiments were performed using the protocol described in Figures 2C-2E. As the IKCa1 and BKCa channels were predominately expressed calcium-activated potassium channels, only their effects in ET-1-induced calcium signaling was investigated here. In these experiments, the pic amplitude of the ET-1-induced calcium mobilization and SOC were quantified and compared between different conditions. As shown in Figures 3C and 3D, treatments of the cells with siIKCa1 or siBKCa (20 nM for 48 h) did not modify significantly the ET-1-induced calcium release from intracellular stores whereas the amplitude of the CCE was decreased by $20 \pm 4.5\%$ vs. CTL (n=140) for siIKCa1 (Figures 3C and 3E).

Similar results were also obtained with siBKCa, where the ET-1-induced calcium release from intracellular stores was not significantly modified whereas the amplitude of the CCE was decreased by $70 \pm 6.5\%$ vs. CTL (n=110) (Figures 3D and 3F). However, in siIKCa1-transfected cells, the second phase of the CCE amplitude was inhibited by $39 \pm 8\%$ vs. CTL (n=140), suggesting the involvement of the IKCa1 in first and second phase of the ET-1-induced CCE in PC-3 cells.

These results suggest that IKCa1 and BKCa channels regulate the CCE induced by ET-1 in PC-3 cells. These observations were also confirmed by the use of the pharmacological inhibitors, TRAM-34 (10 μ M) for IKCa1 and paxilline (10 μ M) for BKCa channels. Next, the co-implication of IKCa1 and BKCa channels in the ET-induced CCE was studied by using the pharmacological inhibitors, TRAM-34 (10 μ M) for IKCa1 and paxilline (10 μ M) for BKCa, channels. These experiments were performed on the cells treated either by siControl (siCTL) or siIKCa1 or siBKCa. After siRNA treatments for one potassium channel type, the ET-1-induced calcium entry was elicited to evaluate the contribution of each potassium channel (IKCa1 or BKCa) in ET-1-induced CCE. Then, the inhibitor of the other calcium-activated channel was applied on the remaining CCE to evaluate the contribution of the channel (Figure 3D). These experiments showed that both IKCa1 and BKCa were involved in the ET-1-induced CCE in these cells (Figure 3D).

Identification of Ca²⁺ channels involved in ET-1-induced CCE

We previously showed that IKCa1 regulate the cell prostate cancer

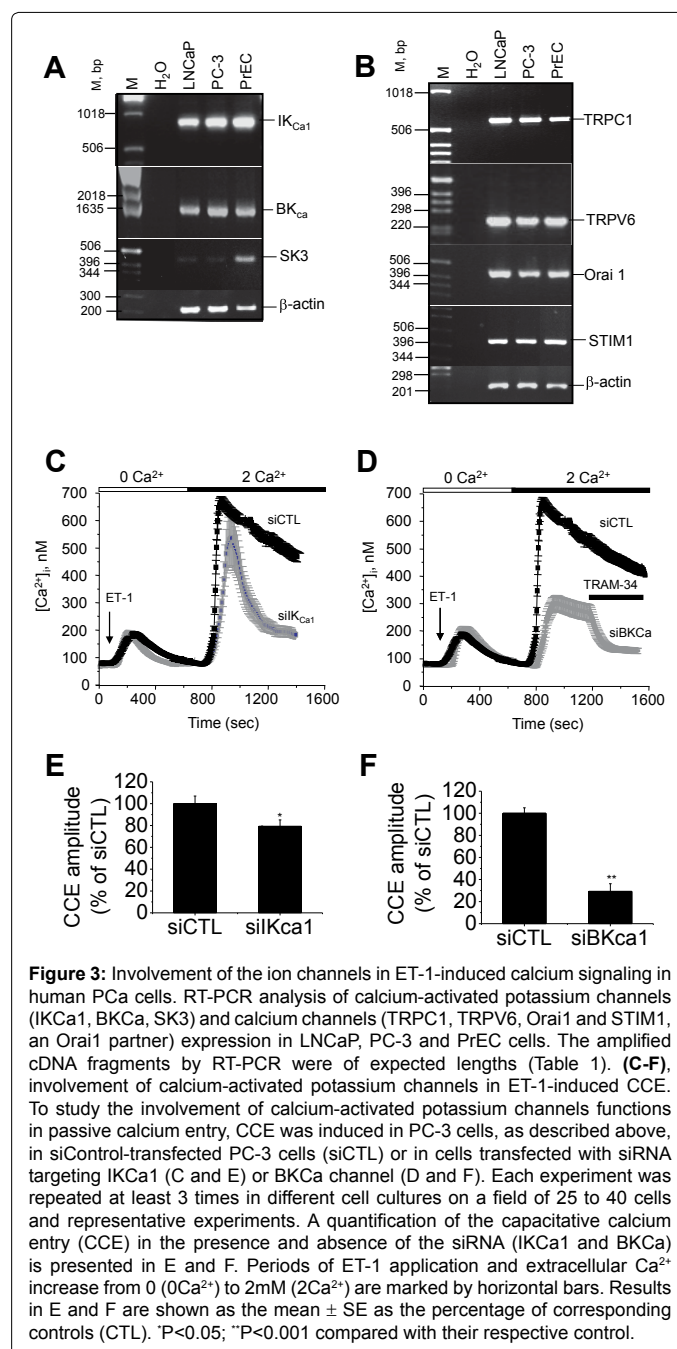


Figure 3: Involvement of the ion channels in ET-1-induced calcium signaling in human PCA cells. RT-PCR analysis of calcium-activated potassium channels (IKCa1, BKCa, SK3) and calcium channels (TRPC1, TRPV6, Orai1 and STIM1, an Orai1 partner) expression in LNCaP, PC-3 and PrEC cells. The amplified cDNA fragments by RT-PCR were of expected lengths (Table 1). (C-F), involvement of calcium-activated potassium channels in ET-1-induced CCE. To study the involvement of calcium-activated potassium channels functions in passive calcium entry, CCE was induced in PC-3 cells, as described above, in siControl-transfected PC-3 cells (siCTL) or in cells transfected with siRNA targeting IKCa1 (C and E) or BKCa channel (D and F). Each experiment was repeated at least 3 times in different cell cultures on a field of 25 to 40 cells and representative experiments. A quantification of the capacitative calcium entry (CCE) in the presence and absence of the siRNA (IKCa1 and BKCa) is presented in E and F. Periods of ET-1 application and extracellular Ca²⁺ increase from 0 (0Ca²⁺) to 2mM (2Ca²⁺) are marked by horizontal bars. Results in E and F are shown as the mean $\pm$ SE as the percentage of corresponding controls (CTL). *P<0.05; **P<0.001 compared with their respective control.

cell proliferation by association with a calcium channel of the TRP family, the TRPV6 [19]. We previously also showed that TRPV6, TRPC1 and Orai1/STIM1 complex could be implicated in CCE in human prostate cancer cell lines [19,30-32]. In order to assess the involvement of these channels in the calcium entry induced by ET-1, we used the siRNA technology to down-regulate TRPC1, TRPV6 and Orai1 expression in PC-3 cells. The use of these siRNA were first validated for their effects on the down-regulation of the TRPV6, TRPC1 and Orai1 expression in prostate cancer cells whereas non-targeting siRNA (siCTL) had no effect on their expression (Figures 4A-4C).

As shown in Figure 5, the siTRPV6 (20 nM) or the siTRPC1 (20 nM) alone for 48h induced a moderate inhibition of the ET-induced CCE in PC-3 cells (Figures 5A and 5B and insets) whereas the

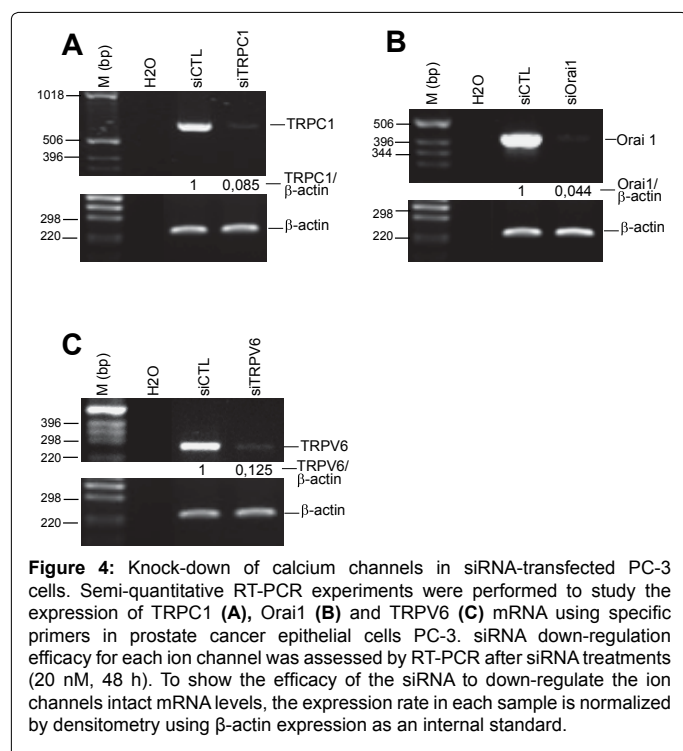


Figure 4: Knock-down of calcium channels in siRNA-transfected PC-3 cells. Semi-quantitative RT-PCR experiments were performed to study the expression of TRPC1 (A), Orai1 (B) and TRPV6 (C) mRNA using specific primers in prostate cancer epithelial cells PC-3. siRNA down-regulation efficacy for each ion channel was assessed by RT-PCR after siRNA treatments (20 nM, 48 h). To show the efficacy of the siRNA to down-regulate the ion channels intact mRNA levels, the expression rate in each sample is normalized by densitometry using β -actin expression as an internal standard.

combined treatments of the cells by both siTRPV6 and siTRPC1 (10 nM each) reduced by about 50% of the ET-induced CCE PC-3 (Figure 5C and inset) cells ($n=135$). In these experiments, the ET-1-induced calcium release from intracellular stores was not significantly modified. The increased inhibition rate of the ET-induced CCE by the combined siTRPV6 and siTRPC1 can result from the homo- and heterotetrameric associations of these channels to form the CCE channels. As TRPC1/TRPV6 channels are involved in only about 50% of the ET-induced CCE, the remaining 50% of the CCE involves another channel probably Orai1 protein which has been proposed to form SOCE channels in prostate cancer cells [32,33].

Involvement of the ion channels in ET-1-induced cell proliferation

After the identification of the potassium (IKCa1 and SK3) and calcium (TRPC1, TRPV6 and Orai1) channels in ET-induced CCE, we studied the involvement of these ion channels in ET-induced cell growth in human PCa cells.

For these experiments, we used pharmacological tools to inhibit the IKCa1 (TRAM-34, 10 μ M), the BKCa (Paxilline, 10 μ M) potassium channels or the calcium channels involved in SOCE (SKF96365, 10 μ M). First the experiments were performed on PC-3 showed that TRAM-34 (10 μ M) and SKF96365 (10 μ M) inhibited the basal cell growth by 42% and 63% respectively, suggesting the involvement of IKCa1 and SOC channels in basal cell growth (Figure 6A). In these experiments, the BKCa channel inhibitor (Paxilline) failed to affect the basal cell growth in PC-3 cells. When the ET-1 was added in the culture medium in the presence of the different inhibitors, we observed that the ET-induced cell growth in PC-3 cells was inhibited by IKCa1 (TRAM-34), the BKCa (Paxilline) potassium channels and by the inhibitor of the calcium channels involved in CCE (SKF96365) (Figure 6A). These experiments suggest that the identified potassium (IKCa1 and BKCa) and calcium (TRPC1, TRPV6 and Orai1) channels form a functional complex to

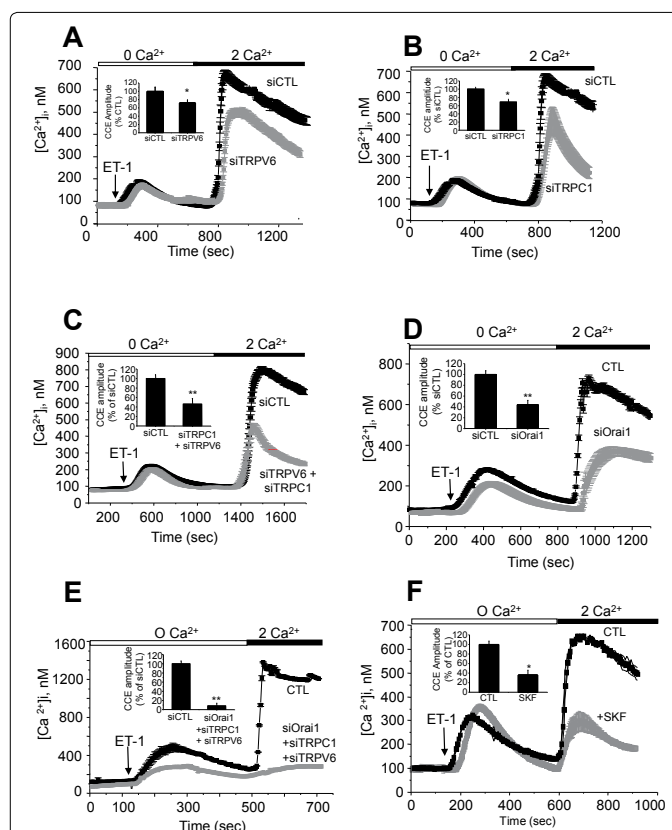
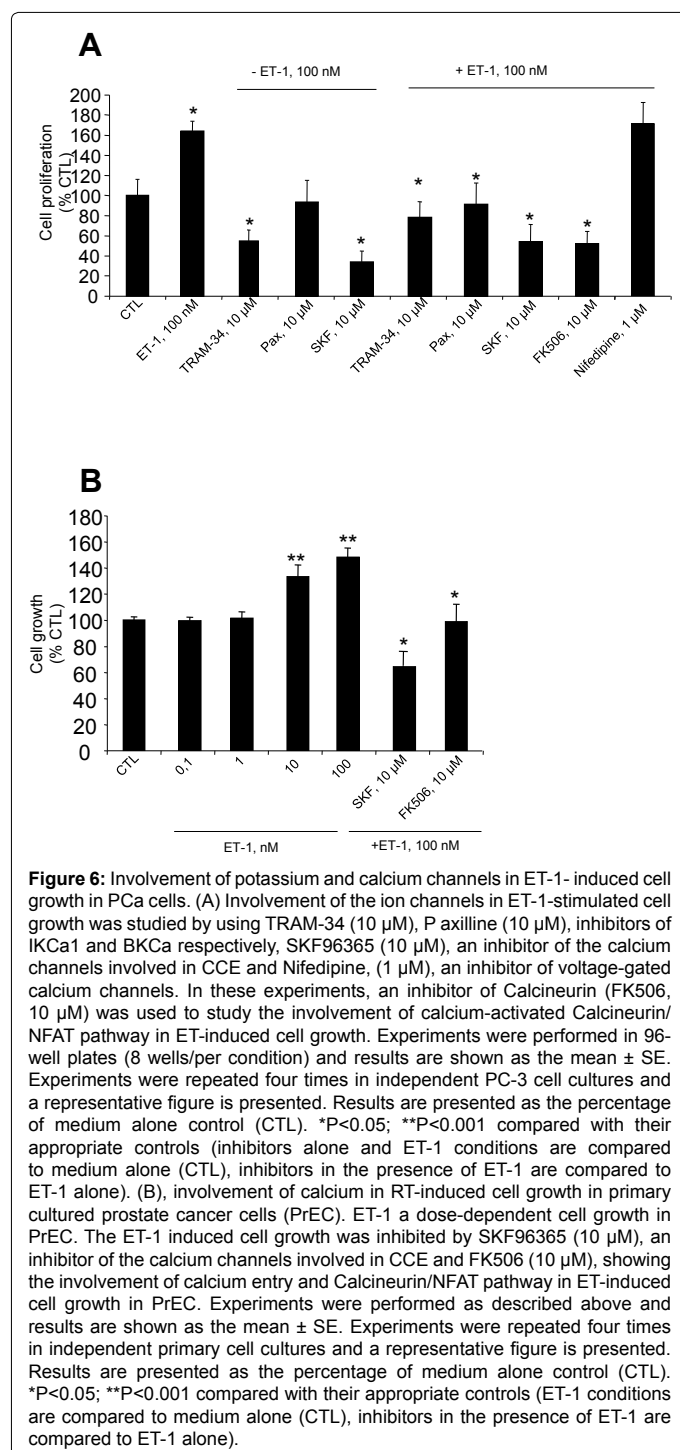


Figure 5: Involvement of the TRP calcium channels in ET-1-induced CCE. To study the involvement of calcium channels functions in passive calcium entry, CCE was induced in PC-3 cells, as described above, in siControl-transfected PC-3 cells (siCTL) or in cells transfected with siRNA targeting TRPV6 (A), TRPC1 (B), TRPV6 and TRPC1 (C), Orai1 (D), TRPV6, TRPC1 and Orai1 (E). The ET-induced CCE was also studied in the absence (control, CTL) or presence of the inhibitor of the calcium channels involved in CCE (SKF96365, 10 μ M) (F). Each experiment was repeated at least 3 times in different cell cultures on a field of 25 to 40 cells and representative figures are presented for each experiment. A quantification of the capacitative calcium entry (CCE) for each experiment is presented in inset. Periods of ET-1 application and extracellular Ca^{2+} increase from 0 (0Ca^{2+}) to 2 mM (2Ca^{2+}) are marked by horizontal bars. Quantifications in inset are the mean $\pm$ SE of the percentage of corresponding controls (CTL). * $P<0.05$; ** $P<0.001$ compared with their respective controls.

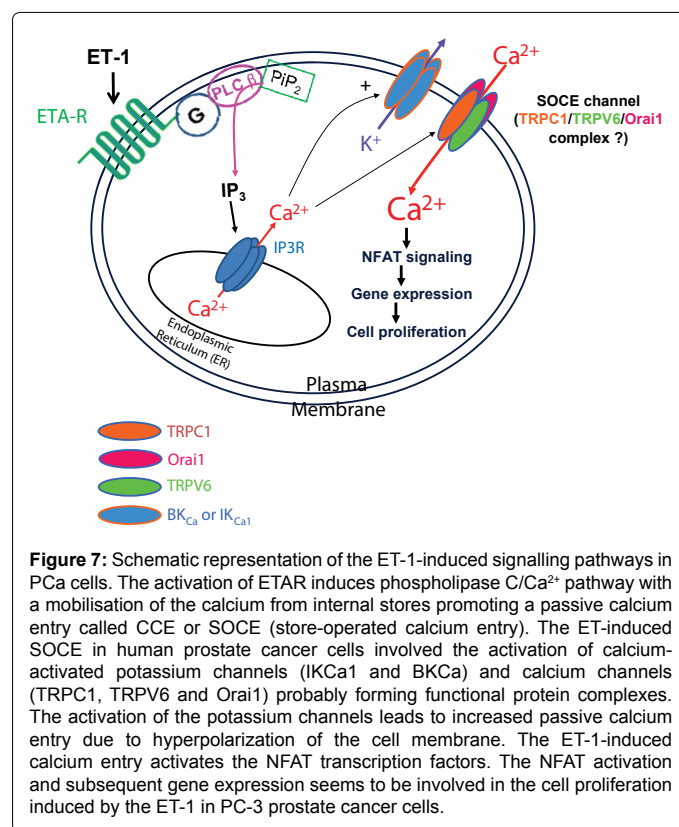
increase the driving force for the calcium entry (IKCa1 and BKCa) via the calcium channels (TRPC1, TRPV6 and Orai1).

To address the participation of Orai1 channels in the remaining 50% of the CCE not inhibited by the siTRPV6 and siTRPC1 treatments, cells were treated with siOrai1 alone (20 nM, 48 h) or in combination with siTRPV6 and siTRPC1. As shown in Figure 5D and inset, in siOrai1 (20 nM, 48 h)-treated cells, the amplitude of the CCE was decreased by $50 \pm 7\%$ vs. CTL ($n=151$) in PC-3 cells (Figure 6). These data suggest that 50% of the CCE induced by ET-1 in PC-3 involve the functionality of Orai-1 channel. Taken together, these results suggest that in PC-3 cells, the TRPC1, TRPV6 and Orai1 are involved in the ET-1-induced CCE by forming homo- or multi-tetramers. These different possibilities of ion channels complex formations in PC-3 cells need further investigations.

Similar experiments were performed in primary cultured PCa epithelial cells PrEC. As shown in (Figure 6B), ET-1 induced a dose-dependent cell growth which was inhibited by the inhibitor of the calcium channels involved in CCE (SKF96365).



One of the way by which Ca^{2+} may act to affect cell proliferation is the activation of NFAT transcription factor. The well-known calcium-sensitive phosphatase calcineurin is a CaM-Ca^{2+} -activated phosphatase, activated by an increase of $[\text{Ca}^{2+}]$. After activation, the phosphatase dephosphorylates the NFAT transcription factor and induces its translocation in the nucleus to activate the gene transcription. A number of activated-NFAT transcription factor are known to favour the cell proliferation by inducing the transcription of genes involved in cell growth. To explore the involvement of Ca^{2+} and the calcineurin/NFAT pathway in ET-1-induced prostate cancer cell proliferation, we used



calcineurin inhibitors, FK506 and Cyclosporin A. When the prostate cancer cells were treated by FK506 (10 μM), the cell proliferation induced by ET-1 was completely blocked in PC-3 cells (Figure 6A) and in PrEC cells (Figure 6B).

Similar results were obtained in PC-3 cells by using the cyclosporine A (data not shown). These data suggest that the increase in free cytosolic calcium concentration is implicated in the mitogenic effect of ET-1 by activating the Ca^{2+} /Calcineurin/NFAT pathway (Figure 7).

Discussion

The ET-1 axis has recently been identified as contributing to prostate cancer progression by inducing cell proliferation [34], apoptosis protection, angiogenesis, and invasiveness. ET-1 has been also shown to be mitogenic and to act as an autocrine or paracrine regulator of growth in many tumor cells [2,35] and anti-apoptotic in prostate cancer [36]. The ETAR is thus actually considered as a new therapeutic target in drug design for the prostate cancers treatments. Ion channels play important roles in several cellular functions such as excitability, contraction, cell cycle progression and metabolism and the link between ion channels and disease has received widespread attention in the last decade. The activation of the ET-1 axis was shown to involve the intracellular Ca^{2+} signalling pathways implicating calcium and potassium channels [4,8,32]. However, the cell signalling involved in the ET-1 transduction pathways remain unknown in human prostate cancer cells.

In the present work, we studied for the first time the effects of ET-1 on proliferation of the human prostate cells and the mechanisms by which activation of ETRs may promote the growth of these cells. We show that the application of ET-1 caused a dose-dependent cell proliferation and an increase in intracellular free Ca^{2+} concentrations ($[\text{Ca}^{2+}]$) via a mobilisation of the internal calcium stores and by a capacitative

calcium entry (CCE) in LNCaP and PC-3 prostate cancer cells. These effects of ET-1 were completely abolished by BQ123, a selective ETAR antagonist, but not by BQ788, a selective ETBR antagonist. By use of the siRNA targeting calcium-activated (IKCa1 and BKCa) potassium channels and calcium channels (TRPC1, TRPV6, Orail1), we showed that these ion channels play an important role in calcium entry induced by ET-1 in PCa cells. These results suggest that these ion channels evidenced here may provide potential targets to block the ET axis in prostate cancers.

Among the Ca^{2+} -activated K^{+} channels family, BKCa and IKCa1 may contribute to cell proliferation and migration. We have recently showed that the intermediate-conductance Ca^{2+} -activated K^{+} channels (IKCa1) is implicated in prostate cancer cell proliferation. Moreover, a recent study has shown that the maxi-conductance Ca^{2+} -activated K^{+} channels (BKCa channels) may play an important role in the progression of human cancer [37]. These results indicate that BKCa channels play an important role in regulating proliferation of human ovarian cancer cells and may induce apoptosis through induction of p21Cip1 expression in a p53-dependent manner.

The ET-1 axis has recently been identified as contributing to prostate cancer progression by inducing cell proliferation [34], apoptosis protection, angiogenesis, and invasiveness, indicating that the pharmacologic inhibition of the ETR signaling pathways may constitute interesting new targets in PCa treatments. ET-1 has been shown to be mitogenic and to act as an autocrine or paracrine regulator of growth in many tumor cells [2,35]. As PCa cells are shown to secrete ET-1, this factor may exert its mitogenic effect in these cells through autocrine mechanisms. A criterion for autocrine growth regulation is an inhibition of spontaneous growth by selective receptor antagonists that specifically block the biological action of the endogenous growth-promoting ligand. Data obtained in this study do support such a view. The results of the present study showed that spontaneous proliferation of unstimulated PC-3 cells was inhibited by the ETAR antagonist BQ123, but not by ETBR antagonist BQ788, suggesting that ET-1 is an autocrine growth factor involved in the regulation of prostate cancer cell proliferation. ET-1 and ETAR not only influence cell proliferation directly but also act synergistically with polypeptide growth factors that are relevant to cancer progression, such as EGF and its receptors [38-40]. ET-1 is known to exert its action through activation of ETAR and ETBR, both of which are expressed in many tumor cells. The mitogenic contributions of the ET receptor subtypes are different in different types of tumors. Several groups have demonstrated that in epithelial tumors *in vitro*, the ET-1-induced mitogenic effect is mediated via the ETAR and in nonepithelial tumors *in vitro* ETBR is involved in this process [17,35,39,41]. The present study showed that even though both ETAR and ETBR are found in LNCaP and PC-3 cells, the mitogenic effect and calcium signaling of ET-1 these cells were mediated predominantly by ETAR. This is evident from the fact that the proliferation and increase of $[\text{Ca}^{2+}]_i$ induced by ET-1 (100 nM) could be blocked by BQ123, a selective ETAR antagonist, but not by BQ788, a selective ETBR antagonist. These results are consistent with previous studies showing that the major function of ETAR is linked to mitogenic signal transduction [35] and [2]. However, the specific function of ETBR expressed in prostate cancer cells remains unknown. It has been proposed in lung cancer that ETBR exerts a variety of compensatory activities, including ET-1 clearance, inhibition of ET-1 secretion, and activation of signal transduction pathways that feedback-regulate the effects of ET-1 [42]. Moreover, a previous study showed that the ETAR-mediated mitogenic and proliferative effect of ET-1 could be enhanced by methylation-induced inactivation of ETBR, leading to the loss

of its unique negative regulatory functions [5]. Further investigation is needed to explore the biological role of ETBR in prostate cancer. $[\text{Ca}^{2+}]_i$ has been shown to be an intracellular secondary messenger that mediates ET-1-induced mitogenic actions in a variety of cell including endothelial cells and vascular smooth muscle cells [43]. However, little is known about the role of $[\text{Ca}^{2+}]_i$ in ET-1-induced mitogenic effects in tumor cells, and no information is yet available in prostate cancer cells. To obtain direct evidence, we first determined the effect of ET-1 on $[\text{Ca}^{2+}]_i$ and then explored its role in the ET-1-induced mitogenic effects in PC-3 and primary-cultured PCa epithelial cells. The $[\text{Ca}^{2+}]_i$ response typically consisted of a spike phase followed by a prolonged plateau phase and was blocked by the ETA-selective receptor antagonist BQ123, which further indicates that the ETAR expressed by human prostate cancer cells is a functional receptor that is linked to the intracellular Ca^{2+} signalling pathway. Data from this study are consistent with the previous observations in human ovarian carcinoma cells [17,44], human cervix (HeLa) and larynx (Hep-2) cells [7]. In such cells, the functional activity of the ETAR was indicated by the ability of ET-1 to induce dose-dependent increases in $[\text{Ca}^{2+}]_i$, which are inhibited by the specific ETAR antagonist, BQ123. It is well established that the elevation of $[\text{Ca}^{2+}]_i$ induced by ET-1 and ETAR mainly results from Ca^{2+} influx and Ca^{2+} release from intracellular Ca^{2+} stores via the PLC/phosphoinositol/ Ca^{2+} pathway. To further determine the role of $[\text{Ca}^{2+}]_i$ in ET-1-induced proliferation of LNCaP and PC-3 cells and to identify possible alternative Ca^{2+} pathways, we investigated two different mechanisms by using a PLC inhibitor (U73122) and potassium and calcium channel blockers. The ET-1-induced cell proliferation (Figure 1) and increases of $[\text{Ca}^{2+}]_i$ at 100 nM (data not shown) were significantly reduced by U73122, the specific inhibitor of PLC, but not by U73433, its inactive analogue.

Chelating intracellular Ca^{2+} reduced the ET-1-induced mitogenic effects PC-3 and PrCE cells. These data indicate that the ET-1 induced elevation of $[\text{Ca}^{2+}]_i$ and that the Ca^{2+} release from intracellular stores via activation of the phospholipase C/phosphoinositol/ Ca^{2+} pathway [1]. The $[\text{Ca}^{2+}]_i$ plays an important role in the ET-1-induced cell proliferation. The results of the present study are compatible with the importance of Ca^{2+} signalling and related responses in the ET-1-induced mitogenesis in vascular smooth muscle cells [43] and the human ovarian carcinoma cells [17]. Taken together with the findings presented above, we provide the first evidence that $[\text{Ca}^{2+}]_i$ plays an important role in the ET-1-induced cell proliferation in prostate cancer cells. Number of studies has shown that the ET-1 effects on cell proliferation of osteoblasts involve the activation of the calcineurin/NFAT pathways leading to the nuclear translocation of NFAT1, [45]. Our results obtained by using an inhibitor of the Calcineurin (FK506) suggest the involvement of the calcineurin/NFAT pathways in ET-1-induced cell growth in human prostate cancer cells.

Since it was reported that intracellular downstream effectors for direct ET-1-induced proliferative action, including PKC, EGF, insulin-like growth factor-1 and mitogen-activated protein kinase (MAPK), have been identified to various degrees in a number of cancers [38,46,47], it is likely that the molecular mechanisms that underlie this activity are varied in different tumors. Further studies are required to explore whether the Ca^{2+} -independent signalling pathway is involved in this process.

Increased ETAR expression is also seen in both primary and metastatic prostate cancer, correlating positively with advancing tumor stage and grade. The results of this study showed that mRNA of ETAR and ETBR are expressed in the human prostate cancer cells LNCaP

and PC-3. We are finding in this study that ET-1 increase $[Ca^{2+}]_i$ in the prostate cancer cells androgeno-dependent LNCaP and androgeno-independent PC-3 and that the $[Ca^{2+}]_i$ plays an important role in the mitogenic effect of ET-1 in prostate cancer cells.

This increase of the $[Ca^{2+}]_i$ is inhibited by the agonist of the ETAR (BQ123) demonstrating that this mechanism passes by the activation of ETAR. We have studied the capacitive calcium entry (CCE) induced by the ET-1. However we observe a CCE whose amplitude is 3 times superior at the increase of calcium that one observes in condition $2Ca^{2+}$. However, when the cells are placed in a medium without calcium, we observed an increase in the amplitude of the calcium response corresponding to the mobilization from internal stores and to the CCE. These observations could be due to the fact that the calcium channels implied in this CCE in response to ET-1 are translocated to the plasma membrane when the cells are incubated in a without calcium. In the present studies, using the pharmacological tools and the siRNA technology, we identified the calcium-activated potassium channels and the calcium channels involved in CCE induced by ET-1 in prostate cancer cells. As the calcium entry appear to be a pathway involved in the effects of the ET-1 on the prostate cancer cells, these channels could constitute the targets in the development of the therapeutic strategies targeting the ET-1 axis in prostate cancer.

ET-1 production by prostate cancer cells is down-regulated by androgens and up-regulated by factors involved in tumour progression indicating a role for this peptide in the biology of prostate cancer [48]. The ET-1 is overexpressed with prostate cancer evolution so this peptide can promote cancer proliferation. Overexpression of the ETAR has been found in a number of human cancer cell lines. Activation of the ETAR by endothelin-1 (ET-1) promotes cell proliferation and survival in these tumours, whereas activation of the ETBR results in an opposing effect. Therefore, blockade of ETAR may have antitumor effects, while sparing ETBR-mediated effects such as induction of apoptosis and clearance of ET-1. However, a study showed that the inhibition of the ETBR, and not the ETAR, inhibited the growth of the cancerous cells of skin [49]. Actually, the ET-1 axis is considered as a therapeutic target in cancer. The discovery of the ET-1 axis components and their function in human cancer has propelled the development a number of different approaches to target them. One of the most promising approaches is represents by the use of potent and selective ETAR, ETBR, or mixed ETAR/ETBR antagonists, which enhance tumor perfusion, thus potentiating the therapeutic efficacy of anticancer agents. The critical role of ET-1 axis and the therapeutic relevance of ET-1 receptor antagonists in a range of malignancies have led to a new generation of molecularly targeted therapies for cancer. Clinical testing of atrasentan, ETAR antagonist, has demonstrated benefit in prostate-specific antigen (PSA) progression, markers of bone turnover, and decreases pain in men with prostate cancer, but has not demonstrated significant improvement in survival or time to cancer progression [14]. The ET-1 axis has recently been identified as contributing of lot of cancer as prostate cancer [34]. The endothelin-converting enzyme (ECE-1) is overexpressed in prostate cancer [50]. In the noncancerous prostate gland, ET-1 is produced by epithelial cells, the highest concentration of ET-1 being found in the seminal fluid. In prostate cancer, key components of the ET-1 clearance pathway, such as ETBR, are diminished, resulting in an increase in local ET-1 concentrations. Increased ETAR expression is also seen in both primary and metastatic prostate cancer, correlating positively with advancing tumor stage and grade. Actually, Atrasentan is a potent, oral, selective endothelin-A (ETA) receptor antagonist that has clinical activity in patients with hormone-refractory prostate cancer (HRPC). The authors of this study report the results from a phase 3, randomized,

double-blind, placebo-controlled trial of atrasentan in patients with non-metastatic HRPC [15].

Accumulating data have shown that increases in intracellular free Ca^{2+} play a major role in many cellular processes. The deregulation of Ca^{2+} signalling is a feature of a variety of diseases, and modulators of Ca^{2+} signalling are used to treat conditions as diverse as hypertension to pain. The Ca^{2+} signal also plays a role in processes important in cancer, such as proliferation and migration. Many studies in cancer have identified alterations in the expression of proteins involved in the movement of Ca^{2+} across the plasma membrane and subcellular organelles. In some cases, these Ca^{2+} channels or pumps are potential therapeutic targets for specific cancer subtypes or correlate with prognosis [51]. A remodelling of calcium homeostasis can occur in cancer cells. Although alterations in Ca^{2+} signalling may not be a requirement for the initiation of cancer, the consequences of altered calcium transport in cancer cells may be significant and contribute to tumor progression. Characterizing such changes may help to identify new therapeutic targets. The influx of calcium across the plasma membrane into the cell is a key trigger or regulator of cellular processes relevant to tumor progression, including proliferation, migration, and apoptosis. Ca^{2+} -permeable ion channels of almost every class have now been associated with aspects of tumor progression [52]. During carcinogenesis, Ca^{2+} signalling of some malignant cells is significantly remodelled in a way that compromises normal physiological functions at the same time enabling them to overwhelm normal cells by giving them unconditional advantages for uncontrolled multiplication, evasion of programmed cell death, adaptation to oxygen and nutrients sparse conditions, invasion and spreading beyond the primary tumour site [52]. Nevertheless, as malignant remodelling of Ca^{2+} signalling helps to sustain cancer hallmarks, learning its intimate mechanisms and identifying the molecular players involved poses an opportunity for therapeutic halting the progression of certain hallmarks or even reversing them.

Several mechanisms are shown to be involved in the regulation of ion channels expression in cancer: tumor Hypoxia [53,54], Promoter methylation [55,56], REST (repressor element 1-silencing transcription factor) transcription factor expression [57,58], Androgen [21] and Estrogen Receptor transcription activity [59] and the function of p53 protein in cancer. In the latter context, the authors [60] showed that ion channel genes are differentially expressed with a change in p53 mutation status, ER status, and tumor histological grade of breast cancer. In these studies, five ion channel genes were upregulated in p53 mutant tumors, including *KCNE3*, *KCNN4*, and *MCOLN2*; while 17 ion channel genes were downregulated, including *ANO1*, *KCNMA1*, and *TPCN1*. In the same context, Cheong et al. reported that the gene encoding K(Ca)3.1 calcium activated potassium channel (*KCNN4*) contains a functional repressor element 1-silencing transcription factor (REST or NRSF) binding site and is repressed by REST. These authors showed also that the expression of REST declines when there is cellular proliferation, showing an inverse relationship with functional *KCNN4*. Taken together, these data suggest that the ion channels genes expression is multifactor-dependent and in addition it might be tumor dependent. Recent data showed that the histone methyltransferase G9a also plays a role in the regulation of K(+) channel associated genes [56]. The histone methyltransferase G9a is shown to maintain DNA methylation via interacting with DNA methyltransferases (DNMTs) [61]. Interestingly, aberrant expression of histone methyltransferase G9a have been found in many malignant carcinomas, including prostate cancer, B cell chronic lymphocytic leukemia, colon cancer, hepatocellular and lung cancer [62] which could be a plausible pathway involved in the overexpression

of potassium channels in prostate cancer. The overexpression of the potassium and calcium channels in cancer presents an advantage for cancer cells to modulate the cancer cells membrane potential to promote calcium entry via voltage-independent calcium channels.

Capacitative Calcium Entry (CCE), also named Store-operated Ca^{2+} entry (SOCE), is critical for Ca^{2+} signaling in non-excitable cells and involved in the transduction pathways of activated receptors to hormones, neurotransmitter and growth factors. Responses regulated by ET-1 have been associated with increases in $[\text{Ca}^{2+}]_i$, either by influx of calcium or release of intracellular calcium stores. $[\text{Ca}^{2+}]_i$ is tightly regulated by a multitude of ion channels and exchangers that control influx, efflux, sequestration, and release of calcium [63,64]. However, the involvement of calcium signaling and ion channels in the effects of ET-1 on prostate cancer cells has not been investigated. In the present study, we showed that the activation of the ETAR induced a mobilization of the calcium from internal stores and a passive calcium entry (CCE). We also showed the Ca^{2+} dependency of ET-1-induced proliferation in PCa cells by using the intracellular Ca^{2+} -chelator BAPTA (10 μM) (Figure 1E) where BAPTA abolished ET-1 induced proliferation suggesting an important role played by the ET-1-increased cytosolic calcium increase. Our investigations showed clearly that the ET-induced CCE in human prostate cancer cells involved the activation of calcium-activated potassium channels (IKCa1 and BKCa) and calcium channels (TRPC1, TRPV6 and Orai1). The involvement of these potassium and calcium channels under ET-1 might be due to the formation of a functional complex involved in the calcium entry induced by ET-1. In this complex, by modulating the membrane potential, the functionality of the potassium channels induce an increase in driving force for the calcium entry via the calcium channels. In this context, in a previous work, we showed the expression and functionality of intermediate-conductance calcium-activated potassium channels (IKCa1) in human PCa as well as their involvement in PCa cells proliferation. In this work, the activation of IKCa1 hyperpolarized membrane potential and, by promoting the driving force for calcium, induced calcium entry through TRPV6, a cation channel of the TRP (Transient Receptor Potential) family [19]. In the present study, the activation of ETAR induced a CCE involving these potassium and calcium channels (IKCa1 and TRPV6) participating in the formation of a functional complex activated by the G-protein-coupled receptor (GPCR). In the present study, The ET-1-induced CCE involved also the BKCa potassium channel. The activation of the IKCa1 and BKCa potassium channels by ET-1 receptors activation was previously shown. In porcine coronary artery smooth muscle cells, it has been demonstrated that the activation of ETAR induced an increase in the open probability of BKCa channel [65]. In the same manner, The ET-1 induced the activation of BKCa potassium channels and a calcium entry in HUVEC endothelial cells [66]. In these studies, activation of BKCa by ET-1 induced a capacitative Ca^{2+} influx which plays an important role in ET-1-mediated endothelial cell proliferation. The IKCa1 potassium channel activation by ET-1 has been also shown in astrocytes [67,68]. Similar to our present data, Peng et al. [68] have shown that, by activating ETAR, ET-1 induced the activation of BKCa and IKCa1 by increasing intracellular Ca^{2+} concentration, in human pulmonary vasculature. However, the mechanism of the ET-1-induced activation of BKCa and IKCa1 potassium channels in these cells systems are not known. One of the most plausible explanations would be the activation of the Ca^{2+} -activated potassium channels by the release of the calcium from internal stores induced by ET-1 as shown in the present study. Store-operated Ca^{2+} entry involved in the development of CCE is a critical Ca^{2+} influx pathway and represents the major Ca^{2+} influx mechanism in non-excitable cells [69]. The pathway involves the

activation of Ca^{2+} influx upon intracellular Ca^{2+} store depletion [69]. The canonical components of store-operated Ca^{2+} entry are the calcium influx channel Orai1 and the endoplasmic Ca^{2+} depletion sensor STIM1 (stromal interaction molecule 1) [70]. This pathway has rapidly become one of the Ca^{2+} influx pathways most studied in cancer. In the present study, our data suggest that 50% of the CCE induced by ET-1 in PC-3 cells involve the functionality of Orai1 channels. The recruitment by ET-1 of the calcium channels involved in CCE has been also the subject of several studies in different cell systems. Ohba et al. were among the first to demonstrate a potential role for Orai1/STIM1 in mediating cardiac hypertrophy [71]. They demonstrated that in neonatal cardiomyocytes ET-1 induced activation of CCE, NFAT nuclear translocation, and cardiomyocyte hypertrophy; importantly, however, all were attenuated following knockdown of STIM1, a protein partner of Orai1. Similar to their earlier study [72], they also found that TRPC1 expression was upregulated in response to ET-1; interestingly, this increase was also attenuated following knockdown of STIM1. Despite the important role of Orai1/STIM1 in CCE, several published data show that the latter proteins are able to form functional complexes with other channel proteins. TRPC channels, including TRPC1, TRPC3, TRPC4, have been proposed as possible candidate channels for these protein complexes. TRPC1 is the best characterized channel in this regard and reported to contribute to endogenous SOCE in many cells types. TRPC1-mediated Ca^{2+} entry and cation current in cells stimulated with agonist or thapsigargin were inhibited by low $[\text{Gd}^{3+}]$ and 10-20 μM 2APB (conditions that block SOCE). Importantly, STIM1 also associates with and gates TRPC1 via electrostatic interaction between STIM1 and TRPC1. Further, store depletion induces dynamic recruitment of a TRPC1/STIM1/Orai1 complex and knockdown of Orai1 completely abrogates TRPC1 function. Despite these findings, there has been much debate regarding the activation of TRPC1 by store depletion as well as the role of Orai1 and STIM1 in SOC channel function [73].

In a published work, Lee et al. [74] studied the interaction between STIM1 and Orai1 and the channels of the TRPC family. They reported that the STIM1 Orai1-activating region domain of STIM1 interacts with the TRPC channel coiled coil domains (CCDs) and that this interaction is essential for opening the channels by STIM1. In this work, they also showed that cell stimulation enhanced the formation of Trpc1-Stim1-Trpc3 complexes. These data suggest also that the Orai1/TRPC1/STIM1 complexes would be able to bind to other calcium channels (TRPV6) to form functional molecular complexes. In this context, Schind et al. [75] showed that TRPV6 exhibited substantial co-localization and *in vivo* interaction with TRPC1 in HEK293 cells.

All these data suggest that in PCa cells, a molecular complex exist or could be formed by Orai1/STIM1/TRPC1/TRPV6 under ET-1 treatment to promote a calcium entry in these cells. But this aspect needs further investigations. In addition, this calcium channel complex could be associated with the calcium-activated potassium channels (IKCa1 and BKCa) as we showed in our previous studies [19] concerning the formation of a IKCa1/TRPV6 involved in the PCa cells proliferation. Similar associations were also shown for TRPC1 and BKCa calcium and potassium channels. TRPC1 was shown to physically associate with BKCa in vascular smooth muscle cells (VS.MCs) and that Ca^{2+} influx through TRPC1 activates BKCa to induce membrane hyperpolarization. The hyperpolarizing effect of TRPC1-BKCa coupling could serve to reduce agonist-induced membrane depolarization, thereby preventing excessive contraction of VS.MCs to contractile agonists. Interestingly, Orai1 was also shown to interact with BKCa [76]. In the latter work, the authors showed that Orai1 physically associates with BKCa to form a signaling complex in the rat mesenteric

artery smooth muscle. Ca^{2+} influx via Orai1 stimulates BKCa, leading to membrane hyperpolarization. Taken together our data presented here suggest the formation in prostate cancer cells of an ion channel molecular complex formed of several calcium-permeable (TRPC1, TRPV6 and Orai1) and potassium channels to promote a calcium entry and cell proliferation in ET-1-stimulated cells.

In summary, we showed that the ETAR and ETBR are expressed in the human prostate cancer cells and ET-1 enhances the proliferation of PC-3 and primary-cultured PCa epithelial cells (PrCE) cells through activation of ETAR. The activation of this receptor induced phosphoinositol/ Ca^{2+} pathway with a mobilisation of the calcium from internal stores and a passive calcium entry (CCE). The ET-induced CCE in human prostate cancer cells involved the activation of calcium-activated potassium channels (IKCa1 and BKCa) and calcium channels (TRPC1, TRPV6 and Orai1) (Figure 7). Interestingly, intracellular calcium and the identified ion channels mediated the ET-1-induced cell growth. As the ions channels described here are expressed in prostate cancer, the present data raise the intriguing possibility that they may be considered as a novel therapeutic target for the treatments of prostate cancer.

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Conflict of Interests

The authors declare no conflict of interests.

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