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# Ghrelin inhibits proliferation and increases T-type $Ca^{2+}$ channel expression in PC-3 human prostate carcinoma cells

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ABSTRACT

Ghrelin is a multifunctional peptide hormone with roles in growth hormone release, food intake and cell proliferation. With ghrelin now recognized as important in neoplastic processes, the aim of this report is to present findings from a series of *in vitro* studies evaluating the cellular mechanisms involved in ghrelin regulation of proliferation in the PC-3 human prostate carcinoma cells. The results showed that ghrelin significantly decreased proliferation and induced apoptosis. Consistent with a role in apoptosis, an increase in intracellular free $Ca^{2+}$ levels was observed in the ghrelin-treated cells, which was accompanied by up-regulated expression of T-type voltage-gated $Ca^{2+}$ channels. Interestingly, T-channel antagonists were able to prevent the effects of ghrelin on cell proliferation. These results suggest that ghrelin inhibits proliferation and may promote apoptosis by regulating T-type $Ca^{2+}$ channel expression.


## 1. Introduction

Ghrelin is a 28-amino acid peptide with an *n*-octanoylation indispensable for its biologic activity originally isolated from the stomach as the endogenous ligand for the growth hormone secretagogue receptor (GSH-R) [1,2]. Ghrelin is essentially a multifunctional hormone with roles in growth hormone release, food intake and cell proliferation [2,3]. However, in spite of its widespread and important physiologic actions, its precise regulatory mechanisms remain ambiguous.

The role of ghrelin in the control of cell proliferation and cancer has received considerable attention [3]; however, discordant results have been reported. Hence, proliferative actions of the hormone (or its synthetic analogs) have been observed in hepatoma HepG2 cells, as well as in prostate and breast cancer cell lines [4,5], whereas antiproliferative effects have been reported in non-tumoral thyroid cell lines, as well as in lung, breast and prostate cancer cell lines [6–9]. Although the reason for such discrepancy is unknown, possible explanations could include differences in the methodological approaches, in the cell types investigated and/or the expression of GHS-Rs.

Ghrelin and the GHS-R1a are both present in the prostate [4,10]. However, modified prostatic epithelial cells (both hyperplastic or neoplastic) express higher levels of ghrelin than normal prostate, suggesting the possible existence of an autocrine/paracrine role of the hormone in the neoplastic processes [8,10]. More specifically, in the androgen-independent human prostate carcinoma PC-3 cells, Jeffery and her colleagues reported that ghrelin at nM concentrations stimulated cell proliferation [4,10]. In contrast, Cassoni et al. demonstrated that ghrelin had a biphasic effect on the growth of PC-3 cells [8], with a stimulating activity at low concentrations (10–100 pM) and an inhibiting effect at higher doses (1 μM). It is worth noting however that the molecular mechanism underlying the actions of ghrelin on PC-3 cells proliferation remain virtually unexplored. In the present report, we have examined the role exerted by ghrelin on prostatic cancer cells growth *in vitro* at concentrations quite close to those found in ghrelin-producing tissues (1–50 nM), and further investigated some of the mechanisms by which the hormone was able to inhibit cell proliferation.

## 2. Materials and methods

### 2.1. Materials

[$^3$H]-Thymidine was obtained from Amersham Life Science; fetal bovine serum was purchased from Invitrogen. Ghrelin (55-0-03A) and GHRP-6 (52-1-80B) were purchased from American Peptide Company Inc. Pimozide (EK-400) was from

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Alomone Labs and mibefradil was a gift of Dr. J.C. Gómora (Institute of Cellular Physiology, UNAM, Mexico). All other chemicals were reagent grade purchased from Sigma–Aldrich. Plasticware was from Sarstedt.

### 2.2. Cell culture

Human prostate PC-3 cells were a gift of Dr. A. Zentella (National Institute of Medical Sciences and Nutrition Salvador Zubirán, INCMNSZ, Mexico). They were routinely grown in Advanced RPMI medium (Invitrogen) supplemented with 3% fetal bovine serum, 1% L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in a 5% $CO_2$/95% air humidified atmosphere.

### 2.3. RT-PCR

Total RNA was isolated using TRIZOL (Invitrogen) Quick-RNATM Miniprep (Zymo Research) by a single-step phenol-extraction. Subsequently, cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). PCR for $Ca_V$ channel subunits and β-actin was performed in a single reaction of 20 μL volume using PyroStartTM Fast PCR Master Mix (Fermentas). The latter served as a control following 30 cycles of denaturing at 94 °C for 30 s, annealing at 63 °C for 30 s, and extending at 72 °C for 1 min. Under this PCR condition, the amplification showed linearity as was determined experimentally (data not shown). PCR products were run on agarose gels and visualized by ethidium bromide staining, and the intensities were then measured by scanning the gel with the ChemiDoc XRS Gel Documentation System (Bio-Rad). The $Ca_V$ channel subunits mRNA levels were normalized by house keeping gene β-actin. The primers sets used for the reactions are given in Supplemental Table 1.

### 2.4. Immunocytochemistry

PC-3 cells were fixed in 4% paraformaldehyde and permeabilized with 0.2% Triton X-100. After blocked with normal rabbit serum 10% (Millipore), cells were incubated with goat anti-GHSR1a antibodies (1:500 dilution; sc-10362 Santa Cruz Biotechnology), and then with FITC-conjugated secondary antibodies (Zymed). Cells were then mounted on a glass slide, and viewed on a laser scanning confocal microscope LSM 5 Pascal (Zeiss).

### 2.5. Assays of cell proliferation and apoptosis

Aliquots of PC-3 cells were seeded at a density of $2 \times 10^4$ cells/ml in 24-well culture plates. At ~60% confluence, cells were growth-arrested by serum deprivation for 48 h and were treated with ghrelin for 72 h. Next, 0.01 μCi/well of [$^3$H]-thymidine was added for 6 h. The cells were washed with PBS and precipitated with 5% trichloroacetic acid. After wash with ethanol, incubation overnight in 0.5 ml 0.1 M NaOH and neutralization with 0.1 M HCl, 250 μl of the cell homogenate were placed into polypropylene vials containing 2.5 ml of scintillation fluid. Radioactivity was quantified using a LS6500 scintillation counter (Beckman Coulter). Detection of apoptotic cell death was performed by end-labeling of DNA with TMR red-dUTP, followed by direct analysis of positive cells (TUNEL assay) using the *in situ* Cell Death Detection Kit (Roche Applied Science) according to the manufacturer's protocol. PC-3 cells were growth-arrested, treated as described above and fixed in 4% paraformaldehyde for 1 h at room temperature. Individual nuclei were visualized at ×100 for quantitative analysis. An average of 100 nuclei from random fields was analyzed on each dish.

### 2.6. Analysis of intracellular $Ca^{2+}$ concentration

The free $\left[Ca^{2+}\right]_i$ concentration was measured in trypsinized PC-3 cells loaded with 2 μM 1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2′-amino-5′-methylphenoxy)-ethane-*N*,*N*,*N*,*N*-tetraacetic acid pentaacetoxymethyl ester (fura-2/AM) for 1 h at room temperature in culture medium. Fura-2 fluorescence measurements were performed in a cuvette containing 2 ml of Krebs-Hepes buffer and $\sim 5 \times 10^5$ cells. Fluorescence was monitored with a spectrofluorophotometer by recording excitation signals at 340 and 380 nm and emission signal at 480 nm at 1-s intervals. Maximum and minimum fluorescence values were obtained by adding 1% Triton X-100 and 200 μM EGTA sequentially at the end of each experiment. $\left[Ca^{2+}\right]_i$ was calculated using the formula described by Grynkiewicz et al. [11].

### 2.7. Western blotting

Proteins were resolved in 7% SDS–polyacrylamide gels and transferred to nitrocellulose membranes as described previously [12]. Briefly, after blocking with non-fat milk supplemented with 0.2% Tween 20, membranes were incubated overnight with the primary anti-$Ca_V$3.1 antibodies (ACC-021; Alomone Labs, Jerusalem), washed in TBS-T (10 mM Tris–HCl, 0.15 M NaCl, 0.05% Tween 20), incubated with the secondary antibodies (111-035-003 Peroxidase AffiniPure Goat Anti-Rabbit IgG; Jackson ImmunoResearch Laboratories, Inc.) and developed with the Amersham ECL reagent. As a protein loading control, membranes were stripped and incubated with rabbit anti-calnexin antibodies (sc-11397, Santa Cruz Biotechnology; 208882, Calbiochem). Semi-quantitative analysis was carried out by densitometry using the Kodak digital Science ID v.2.0 system program.

### 2.8. Electrophysiology

$Ba^{2+}$ currents through $Ca_V$ channels were measured using the standard whole-cell patch-clamp technique as previously described [12]. Borosilicate glass electrodes were filled with the internal solution containing (in mM): 125 CsCl, 10 EGTA, 5 $MgCl_2$, 10 HEPES, 4 Na-ATP and 0.1 GTP (pH 7.3). The external solution contained (in mM): 100 TEA-Cl, 40 $BaCl_2$, 10 HEPES and 10 glucose (pH 7.3). Cells were held at a holding potential of −80 mV, and currents were elicited by depolarizing steps ranging from −60 to 70 mV for 140 ms. All the data were obtained and analyzed using an Axopatch 200B (in the whole-cell mode) and the pClamp 8.0 software program (Axon Instruments).

## 3. Results and discussion

As previously described for ghrelin production in prostate cancer, divergent observations have been reported on the GHS-Rs expression in prostate carcinoma cell lines and tissues. Though the presence of GHS-Rs (1a and 1b) at both mRNA and peptide level has been reported in various prostate carcinoma cell lines including PC-3 by Chopin and co-workers [4,10], neither GHS-R1a mRNA nor protein was detected in the same cells by Cassoni et al. [8]. However, despite the absence of GHS-Rs mRNA, the presence of specific [$^{125}$I]Tyr4-ghrelin binding sites was reported in PC-3 cells by the Cassoni group. Moreover, ghrelin binding was displaced by unlabeled ghrelin, synthetic GHS, or unacylated ghrelin, an endogenous ghrelin variant lacking GHS-R1a binding affinity, and therefore these authors suggested the possible expression of an alternative still unidentified ghrelin receptor subtype [8]. In the present report, we first used RT-PCR to re-examine the mRNA-expression of GHS-R1a and 1b in PC-3 cells. As can be seen

in Fig. 1A, single PCR products of the expected size for GHS-Rs types 1a (290 bp) and 1b (310 bp) were generated from cDNA derived from the PC-3 cells, demonstrating unambiguously that both GHS-R isoforms are present in this prostatic cell line.

Likewise, it is well known that the ghrelin actions are exerted by the full-length GHS-R 1a isoform, a protein of ~366 aa with seven transmembrane domains, while GHS-R 1b is predicted to encode a truncated protein of 289 aa with only five transmembrane domains which function is not yet known [13]. We therefore examined the expression and localization of GHS-R 1a on the surface of PC-3 cells. Confocal microscopy detected intense staining for ghrelin type 1a receptor on the plasma membrane and in the cytoplasm (Fig. 1B and D) providing evidence that these cells synthesize the GHS-R 1a protein. As expected, primary antibody-free negative controls failed to stain for the GHS-R 1a specific antibody (Fig. 1C).

The biologic effect of ghrelin on PC-3 cells proliferation was next determined at different concentrations after 72 h of treatment. Fig. 2A shows that ghrelin at concentrations ranging from 10 to 50 nM significantly decreased [$^3$H]-thymidine incorporation into DNA compared to untreated controls. Next, we sought to determine whether ghrelin-induced inhibition of cell proliferation was related to induction of apoptosis. As shown in Fig. 2B, though TUNEL positive nuclei could be found in both control and ghrelin treated PC-3 cells, the number of TUNEL positive nuclei was significantly greater in the later (26.3 ± 0.7% versus 47.7 ± 2%). These results suggest that ghrelin inhibits proliferation and promotes apoptosis of PC-3 human prostate carcinoma cells. In line with this, it has been demonstrated that sustained $Ca^{2+}$ increases can trigger apoptosis in prostate cancer cell lines [14,15]. To verify that this mechanism was involved in the proapoptotic effects of ghrelin, we measured $\left[Ca^{2+}\right]_i$ in fura-2-loaded PC-3 cells by dual-wavelength fluorescence microscopy. Fig. 2C shows that consistent with a role in apoptosis, a significant increase in intracellular free $Ca^{2+}$ levels was observed in the ghrelin-treated cells with respect to the control untreated cells (86 ± 15 versus 43 ± 4 nM).

Interestingly, previous studies have raised the possibility that the expression of voltage-gated $Ca^{2+}$ ($Ca_V$) channels could be regulated by ghrelin [16,17], and given the role these proteins play in cell proliferation [18,19], we next carried out experiments to identify their profile of expression in the PC-3 cells and to test the hypothesis that changes in $Ca_V$ channel expression could be implicated in the decrease in proliferation observed after ghrelin treatment. It is worth mentioning that ten genes coding mammalian $Ca_V$ channels have been reported and that these channels fall into three families distinguished by the membrane potential at which they are activated: (i) $Ca_V1$, encoding high voltage-activated (HVA) L-type channels; (ii) $Ca_V2$, encoding HVA P/Q-type, N-type, and R-type channels; and (iii) $Ca_V3$, encoding low voltage-activated (LVA) T-type channels [20].

Standard RT-PCR was then performed on total RNA to examine what sub-types of $Ca_V$ channels were expressed in PC-3 cells, using sequence-specific primers. Fig. 2D shows a representative gel showing PCR fragments of the predicted sizes detected using primers for the $Ca_V3.1$ and $Ca_V3.2$ sequences. In contrast, no PCR products could be amplified from PC-3 cells RNA when primers for $Ca_V1.2$, $Ca_V1.3$ and $Ca_V3.3$ were used. To our knowledge, this is the first study to report the expression of $Ca_V$ subunit mRNAs in the PC-3 cell line. Potential changes in the levels of $Ca_V3$ mRNAs after ghrelin treatment were subsequently evaluated using semi-quantitative RT-PCR. In these experiments PCR was kept within the exponential phase of the amplification, and a set of experiments were performed to confirm that the relative abundance of the β-actin transcript did not change after treatment with ghrelin. We then estimated the $Ca_V3$ channels band intensities as a fraction of the signal detected for β-actin in the same RNA preparations from PC-3 cells in the control

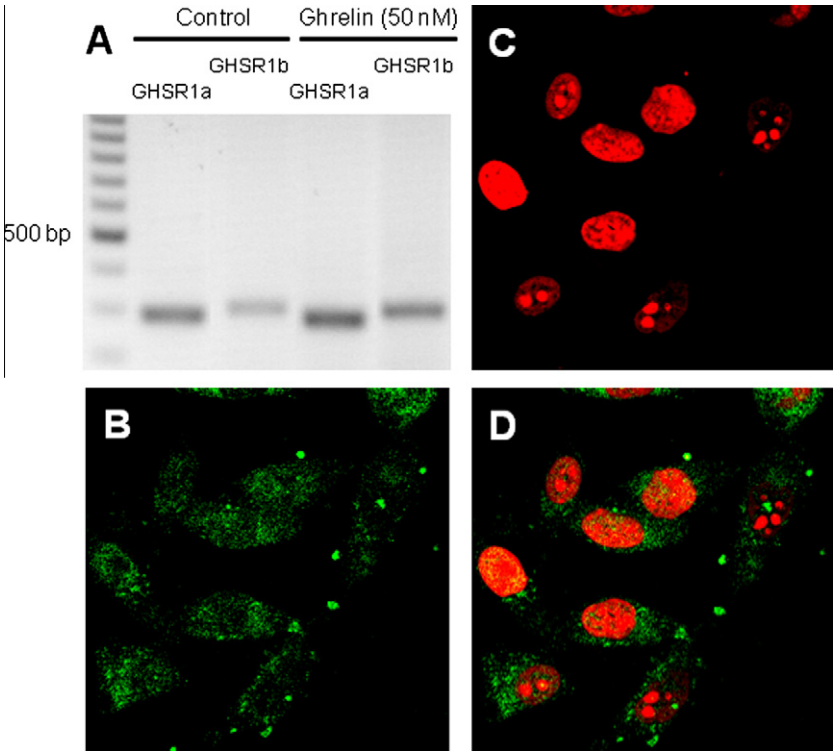


**Fig. 1.** GHSRs expression in the PC-3 prostate cancer cell line. (A) GHS-R 1a and GHS-R 1b RT-PCR products amplified from total RNA. (B) Immunocytochemistry performed using GHS-R 1a specific antibodies. Positive staining is indicated by the green staining. (C) All cell nuclei (shown in red) are non-immunoreactive. (D) Superimposition of images B and C. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

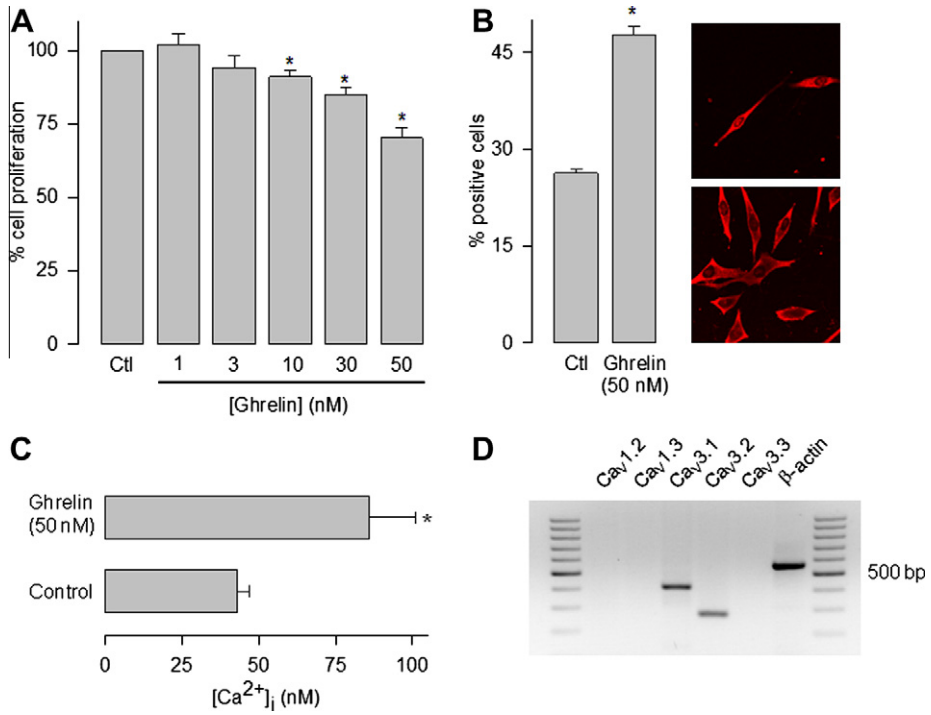


**Fig. 2.** Ghrelin decreases cell proliferation and increases apoptosis as well as cytoplasmic $Ca^{2+}$ levels in PC-3 cells. (A) [$^3$H]-thymidine incorporation into DNA as a function of ghrelin concentration. Cells were treated with ghrelin for 72 h and [$^3$H]-thymidine was included in the last 6 h. (B) Apoptosis was determined for both control and ghrelin-treated cells by the *in situ* TUNEL TMR technique. DNA fragmentation is expressed as % of positive cells per microscopic field. (C) Basal intracellular $Ca^{2+}$ levels measured spectrophotofluorometrically in Fura-2-loaded control and ghrelin-treated cells as described in Section 2. (D) Gel electrophoreses of RT-PCR amplification products for $Ca_V$ channel subunits as listed. Data are the means ± SEM of at least three separate experiments. $^*p < 0.05$, significantly different from untreated controls.

condition and after treatment with ghrelin. The results revealed a small but significant upregulation (~12–15%) in the expression levels of the mRNA for $Ca_V3.1$ (Fig. 3A). Under these experimental conditions, we did not observe apparent differences in $Ca_V3.2$ mRNA levels in the control and the ghrelin-treated cells (Fig. 3B).

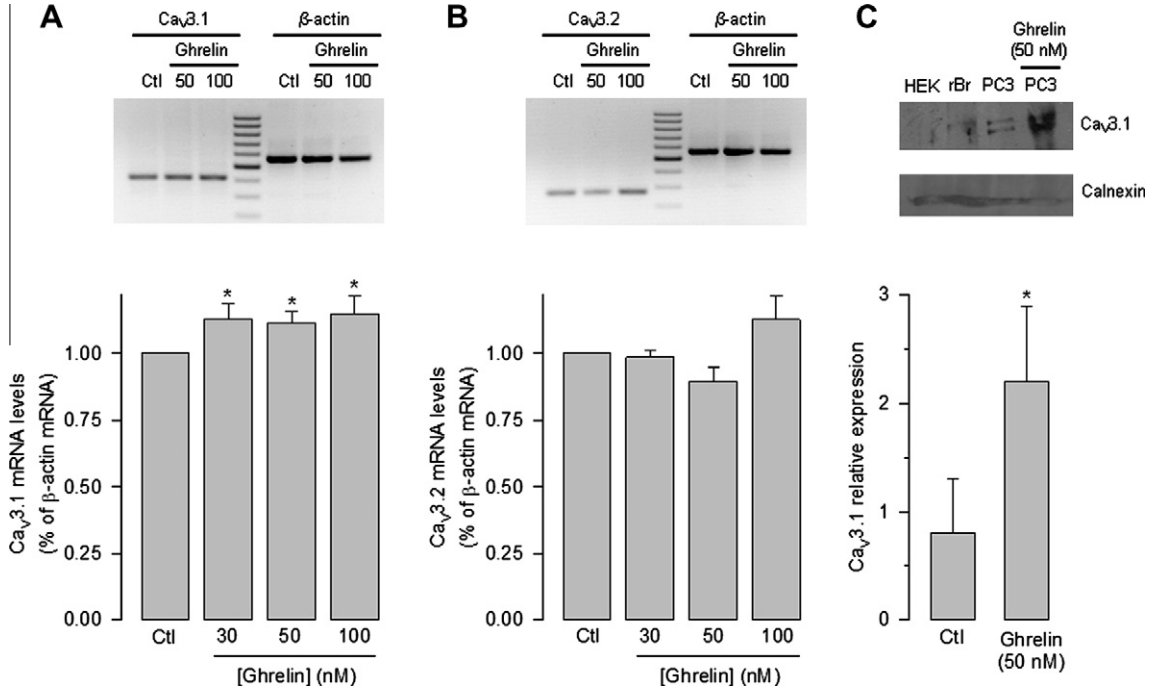


**Fig. 3.** Ghrelin increases $Ca_V3.1$ channel expression in PC-3 cells. Relative quantitative RT-PCR analysis of $Ca_V3.1$ (A) mRNA and $Ca_V3.2$ (B) using β-actin mRNA as a control. The left part of the upper panels represent $Ca_V$ channel subunit mRNA fragments and the right part represents the fragments corresponding to β-actin. The graphs below each blot show average data of the ratio of $Ca_V$ channels to β-actin mRNA density. (C) Representative Western blot (upper panel) and quantitation of the intensity of the bands of $Ca_V3.1$ (lower panel). The value for $Ca_V3.1$ subunit was normalized by the value for the internal standard calnexin. HEK-293 cells (HEK) and rat brain (rBr) served as negative and positive controls, respectively. Values are mean ± SE and $n = 3$. $^*p < 0.05$, significantly different from untreated controls.

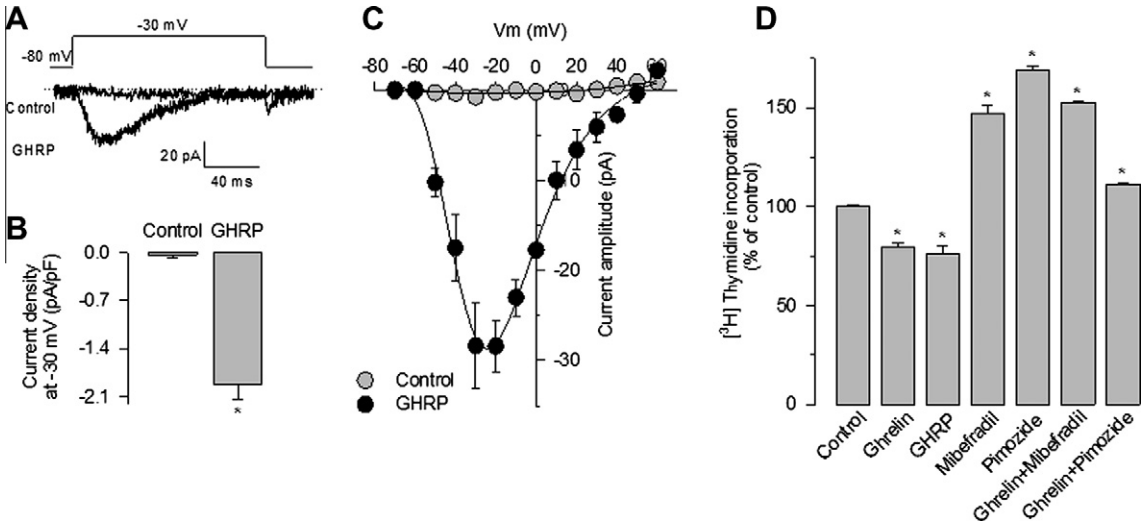


**Fig. 4.** Upregulation of functional T-type $Ca^{2+}$ channels in PC-3 cells by GHRP-6 and their potential role in cell proliferation. (A) Superposition of the whole-cell currents recorded at −30 mV in cells kept in culture in absence (control) and presence of GHRP-6 as an analog of ghrelin. (B) Comparison of current densities for the control and GHRP-treated cells. (C) Current–voltage (*I–V*) relationships for the peak currents shown in A. (D) Effect of blockade of T-type $Ca_V$ channels with pimozide or mibefradil on [$^3$H]-thymidine uptake in cells incubated with ghrelin and GHRP-6. Bars represent the mean ± S.E.M. of at least four different experiments. $^*p < 0.05$, significantly different from untreated controls.

Next, to investigate channel expression at the level of protein, membranes derived from PC-3 cells were screened for the presence of $Ca_V3$ channels using specific antibodies. As can be seen in Fig. 3C, a ~250-kDa band, the expected molecular mass of rat $Ca_V3.1$ was detected in control (rat brain) and PC-3 cells both in presence and absence of ghrelin. The relative levels of $Ca_V3.1$ protein expression were then measured and subsequently analyzed after normalization to those of calnexin. The result of this semi-quantitative Western blot analysis showed that the relative levels of the $Ca_V3.1$ protein in whole cell lysates were increased significantly in the ghrelin-treated cells. In contrast, $Ca_V3.2$ levels were not significantly different between the control and ghrelin treated PC-3 cells (not shown). These data are consistent with the results obtained in the semi-quantitative PCR experiments.

Last, to investigate the functional expression of $Ca_V3$ channels in PC-3 cells, whole-cell patch clamp experiments were performed. Fig. 4A shows that voltage-sensitive currents through $Ca_V$ channels were not detected in any cell from control cultures, even with a very high concentration (40 mM) of the charge carrier ($Ba^{2+}$) in the bath recording solution. In sharp contrast, after ghrelin treatment, PC-3 cells displayed a small, mibefradil-sensitive, rapidly inactivating inward current ranging from −10 to −80 pA with peak at −30 mV (Fig 4C), typical of T-type currents. Thus, T-type currents coincident with changes in the $Ca_V3.1$ mRNA and protein were induced in PC-3 cells after ghrelin treatment.

It is worth mentioning that the unique biophysical properties of T-type $Ca_V$ channels may allow these proteins to carry depolarizing current at low membrane potentials contributing to regulate $[Ca^{2+}]_i$. At low voltages, T-channels are known to mediate a "window current" which refers to the voltage overlap between the activation and steady state inactivation of the channels at low or resting membrane potentials [21]. As a result, there is a sustained inward $Ca^{2+}$ current carried by a small portion of channels that are not completely inactivated. Thus, window current allows T-channels to regulate $Ca^{2+}$ homeostasis under non-stimulated or resting membrane conditions. Interestingly, the inhibitory effect of ghrelin on [$^3$H]-thymidine incorporation was significantly prevented by the $Ca_V3$ channel antagonists mibefradil and pimozide (Fig. 4D), suggesting that $Ca^{2+}$ entry by these channels is a regulatory event of ghrelin-mediated effects on cell proliferation. However, further experiments are needed to investigate whether the increase in $[Ca^{2+}]_i$ produced by the window currents resultant from $Ca_V3$ channel grhelin-induced expression is connected to cell apoptosis.

The present study provides new insights into the role of ghrelin in prostate cancer cell proliferation and the signaling mechanisms involved. In light of our findings, the application of specific inhibitors that block ghrelin signaling might provide new avenues for the design of better adjunctive therapies for prostate cancer.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.10.100.

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