Evidence for specific TRPM8 expression in human prostate secretory epithelial cells: functional androgen receptor requirement

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Abstract

TRPM8 (melastatine-related transient receptor potential member 8), a member of the transient receptor potential (TRP) superfamily of cation channels, has been shown to be a calcium-channel protein. TRPM8 mRNA has also been shown to be overexpressed in prostate cancer and is considered to play an important role in prostate physiology. This study was designed to determine the androgen-regulation mechanisms for TRPM8 mRNA expression and to identify the phenotype of TRPM8-expressing cells in the human prostate. Our findings show that \textit{trpm8} gene expression requires a functional androgen receptor. Furthermore, this article argues strongly in favour of the fact that the \textit{trpm8} gene is a primary androgen-responsive gene. Single-cell reverse transcriptase PCR and immunohistochemical experiments also showed that the \textit{trpm8} gene was mainly expressed in the apical secretory epithelial cells of the human prostate and \textit{trpm8} down-regulation occurred during the loss of the apical differentiated phenotype of the primary cultured human prostate epithelial cells. The androgen-regulated \textit{trpm8} expression mechanisms are important in understanding the progression of prostate cancer to androgen-independence. These findings may contribute to design a strategy to predict prostate cancer status from the TRPM8 mRNA level. Furthermore, as the TRPM8 channel is localized in human prostate cells, it will be interesting to understand its physiological function in the normal prostate and its potential role in prostate cancer development.

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Introduction

Prostate cancer is the most commonly diagnosed neoplasm in men and the second most common cause of cancer-related death in North America and Western Europe. The incidence of prostate cancers increases rapidly with aging. Considering the general aging of the population in industrialized countries, prostate cancer has become a significant health, social, and economic problem. The intensive use of prostate-specific antigen (PSA) screening has led to the improvement of prostate cancer diagnosis at an early stage thus facilitating early treatment. Nevertheless, mortality from prostate cancer has remained relatively constant. In the early stages, prostate cancer cells depend on androgens for growth and survival, so androgen-ablation therapy at this time may be effective in causing a tumour to regress. However, treatment options for advanced hormone-refractory prostate cancers are still relatively inefficient (Martel \textit{et al.} 2003). Indeed, under anti-androgen therapy, prostate cancer and metastasis progress into an androgen-independent stage, causing cancer relapse with a more aggressive phenotype. Consequently, research is required to identify new approaches to diagnosis, prevention, prognostic strategy and/or treatment of prostate cancer. It is therefore
essential to understand what drives the progression to androgen-independence and identify the molecular markers for both the early androgen-dependent and the advanced androgen-independent prostate cancer stages.

It is now evident that many human diseases are linked to ion-channel dysfunctions — i.e. channelopathies. Although channelopathies are well characterized in neuronal, cardiac, and metabolic diseases, much less is known about the role of ion channels in oncogenesis. The family of transient receptor potential (TRP) cationic protein channels, with their extraordinarily diverse activation mechanisms (Clapham et al. 2001), have recently received increasing attention, particularly concerning their links to human diseases (Duncan et al. 1998, Prawitt et al. 2000, Tsavaler et al. 2001). Some of these TRP channels have been reported to be involved in carcinogenesis: TRPM1 (melastatin-related transient receptor potential member 1) in melanoma (Duncan et al. 1998, Fang & Setaluri 2000), and vanilloid-related transient receptor potential member 6 (TRPV6) and TRPM8 in prostate cancer (Peng et al. 2001, Tsavaler et al. 2001). TRPV6 is undetectable in benign prostate tissue and has been shown to be up-regulated in prostate cancer, especially in the later stages with extraprostatic extensions (Fixemer et al. 2003). It has, therefore, been proposed as prognostic marker for tumour progression.

The human trpm8 gene (encoding melastatin-related transient receptor potential member 8, TRPM8), initially known as trp-p8, has been shown to be mainly expressed in the prostate and overexpressed in prostate cancer (Tsavaler et al. 2001). Indeed, trpm8 expression levels have been shown to be highly correlated with prostate cancer tumour stages, leading authors to speculate that trpm8 gene expression was a potential diagnostic marker for prostate cancer (Fuessel et al. 2003, Kiessling et al. 2003). In the meanwhile, two studies demonstrated that both mice and rat orthologues of the human trpm8 gene coded for a functional calcium channel, either in overexpression systems or in rat neurons (McKemy et al. 2002, Peier et al. 2002), although TRPM8’s functions in the prostate are still unknown. Henshall et al. (2003) recently showed that the loss of TRPM8 mRNA expression was associated with a significantly shorter time of PSA relapse-free survival. As the trpm8 gene was silenced in prostate cancer tissue from patients treated preoperatively with anti-androgen therapy, the authors suggested that the trpm8 gene was under androgen control (Henshall et al. 2003). Finally, Zhang & Barrit (2004) used an androgen-sensitive human prostate cell line (lymph node carcinoma of the prostate (LNCaP) cells) to show that trpm8 gene expression was up-regulated by 5α-dihydrotestosterone (DHT). Nevertheless, there is still no evidence for direct regulation of the trpm8 gene by the androgen receptor (AR) in the prostate. Furthermore, although the specific expression of ARs in the intermediate and well-differentiated prostate secretory epithelium is now clearly determined, the phenotype of the TRPM8-expressing cells in the human prostate has not yet been determined. The localization of the TRPM8 channel in prostate epithelial cells may contribute to the understanding of its physiological function in the normal prostate and its potential role in prostate cancer development.

This study was designed to determine the androgen-regulation mechanisms for TRPM8 mRNA expression and identify the phenotype of TRPM8-expressing cells in the human prostate. We demonstrate that AR activation is a key element for the up-regulation of trpm8 gene expression by DHT. Furthermore, our findings indicate that trpm8 is a primary androgen-responsive gene. We also demonstrated the relevance of trpm8-regulation pathways, not only using the widely studied LNCaP androgen-sensitive human prostate cancer and human prostate normal transformed PNT1A (human post-pubertal prostate normal) cell lines, but also in primary cultured epithelial cells or tissue sections taken from resections of human prostates (primary hPCE cells). Finally, we provide direct evidence for TRPM8-channel expression in androgen-sensitive apical epithelial secretory cells as well as in smooth muscle cells of the human prostate.

Androgen-regulated trpm8 expression mechanisms are important in understanding the progression of prostate cancer to androgen-independence and may be helpful in predicting cancer status through TRPM8 mRNA levels.

Materials and methods

Cell culture

LNCaP cells were from the American Type Culture Collection (ATCC). The PNT1A cell line (human post-pubertal prostate normal, immortalized with SV40) was purchased from the European Collection of Cell Cultures (ECACC) and cultured in Dulbecco’s minimal essential medium (DMEM; InVitrogen, CergyPontoise, France) supplemented with 10% foetal calf serum and kanamycin (100 μg/ml). In experiments where the effects of the androgens on gene expression were assessed, LNCaP cells were grown in a steroid-free medium (SFM) for 64 days, and then either used directly for experiments or DHT was added at the desired concentration. Bicalutamide treatments (Casodex; AstraZeneca, London, UK) were performed for 3 days by...
adding a final dose of 10 μM into the complete medium. For protein-synthesis-inhibition experiments, PNT1A cells were transfected by a human AR expression vector (psG5-hARwt) in SFM for 24 h. Then, cells were incubated in a complete medium supplemented with 100 μM cycloheximide (CHX) and 300 μM anisomycin (ANM) for 36 h before harvesting for the reverse transcriptase (RT)-PCR experiments.

**Fresh hPCE cell cultures**

All human specimens of prostate tissue were from patients who had not received anti-androgen therapies previously. In addition, all specimens were diagnosed by anatomopathological examination. After surgery, the conjunctive tissues were eliminated and the epithelial nodules cut into small fragments in DMEM/Ham’s F12 medium containing kanamycin (100 μg/ml), then incubated with 0.1% trypsin in 5 ml DMEM/Ham’s F12 medium for 15 min at 37°C. DNase I (20 μg/ml; Life Technologies) was then added to the medium for 1 min. After enzymatic digestion, the medium was removed and pieces of tissue were incubated for 5 min in each of the following solutions, consecutively: DMEM/Ham’s F12 medium with a trypsin inhibitor (1 mg/ml), Ca²⁺/Mg²⁺-free Hank’s balanced salt solution (HBSS) containing 2 mM EDTA, the same medium containing 1 mM EDTA, and HBSS solution. The cells were mechanically dispersed in the last solution and seeded into 35 mm Petri dishes (Nunc) in KSF medium (Keratinocyte Serum-Free; Gibco-BRL) containing 2% foetal bovine serum and antibiotics (100 μg/ml kanamycin, 100 μg/ml gentamycin), supplemented with 50 μg/ml bovine pituitary extract and 50 ng/ml epidermal growth factor (complete KSF medium).

All experiments carried out on patient tissues were registered under approval number CP 01/33, issued by the Comité Consultatif de Protection desPersonnes dans la Recherche Biomedicale (CCPPRB) in Lille, France.

**Transient expression studies**

PNT1A and hPCE cells were cultured in 60 mm Petri dishes for RT-PCR and 35 mm Petri dishes for single-cell RT-PCR (SCRT-PCR) until 50% confluency. Cells were then transiently transfected for 8 h by 2 μg (in 60 mm dishes) or 1 μg (in 35 mm dishes) wild-type human AR (psG5-hARwt) or a chimera of a mutant human AR and a green fluorescent protein (GFP; p-C1-EGFP-AR-L707R) expression vector, using a Gene Porter™2 Transfection Reagent (Gene Therapy Systems, San Diego, CA, USA) in a serum-free medium. hPCE cells were co-transfected by 0.2 μg pDsRed2-N (BD Bioscience Clontech, Palo Alto, CA, USA), a plasmid vector coding for a red fluorescent protein, and 1 μg psG5-hARwt. The DsRed positive cells were then chosen as the transfected cells for AR. Complete DMEM without steroids was added overnight to the PNT1A cell line and complete KSF medium without steroids to the hPCE cells. A complete medium containing steroids was added 24 h later for the experiment itself.

**Small interfering (si) RNA cell transfection**

PNT1A cells were transfected overnight by psG5-hARwt expression vector, as described above. 12 h later, cells were retransfected by 15 nM siRNA against AR (siRNA-AR), using 15 μl TransIT-TKO transfection reagent (Mirus, Madison, WI, USA) previously mixed with 200 μl free RPMI 1648 for 15 min. The complete mixture was finally added to 2.5 ml complete DMEM in 60 mm dishes.

Ready-to-use siRNA-AR (processing option, A4) was synthesized by Dharmaco (Lafayette, Colorado, USA). The sense sequence of the siRNA used was 5’-GACUCAGCUGCCCCAUCCA(dTdT)-3’.

**Analysis of trpm8 and AR gene expression (semi-quantitative RT-PCR)**

Total RNA was isolated using the guanidium thiocyanate/phenol/chloroform extraction procedure (Chomczynski & Sacchi 1987). After a DNase I treatment to eliminate genomic DNA, 2 μg total RNA was reverse transcribed into cDNA at 42°C using random hexamer primers (Perkin Elmer, Foster City, CA, USA) and murine leukaemia virus (MuLV) RT (Perkin Elmer) in a 20 μl final volume, followed by PCR as described below. The PCR primers used to amplify TRPM8 cDNAs were designed by Tsavaler et al. (2001). The PCR primers used in RT-PCR and SCRT-PCR studies to amplify the RT-generated TRPM8, AR, β-actin, keratin (K)5, K8, K14, and K18 cDNAs, designed on the basis of established GenBank sequences, are listed in Table 1. PCRs were carried out on the RT-generated cDNA using a GeneAmp PCR System 2400 thermal cycler (Perkin Elmer). To detect trpm8 and AR cDNAs, PCR was performed by adding 1 μl RT template to a mixture of (final concentrations): 50 mM KCl, 10 mM Tris/HCl (pH 8.3), 2.5 mM MgCl₂, 200 μM each dNTP, 600 nM sense and antisense primers, and 1 U AmpliTaq Gold (Perkin Elmer), in a final volume of 25 μl. DNA amplification conditions included an initial 5-min denaturation step at 95°C (which also activated the Gold variant of Taq DNA polymerase).
polymerase) and 40 cycles of 30 s at 95°C, 30 s at 58°C, and 40 s at 72°C, and finally 5 min at 72°C. Semi-quantitative PCR assays were carried out under non-saturating conditions: 35 cycles for TRPM8 and 27 cycles for β-actin. Then, density was measured using Quantity one software (BioRad) and the data were analysed using Origin 5.0 (Microcal Software, Northampton, MA, USA).

SCRT-PCR
The SCRT-PCR studies were carried out as described previously (Roudbaraki et al. 1999). Briefly, primary cultured cells were chosen either randomly (freshly isolated primary-culture cells) or by detecting DsRed2 fluorescence in co-transfected hPCE cells with pDsRed2-N vector and psG5-hARwt, and the cytoplasm of the individual cells was collected using the patch-clamp technique. Patch pipettes were pulled from heated (200°C, overnight) borosilicate glass tubing filled with the internal pipette solution (10μl of 140 mM KCl, 2 mM MgCl2, 1.1 mM EGTA, and 10 mM Hepes, pH 7.25). The pipette cytoplasm content (approximately 8μl) was ejected into an Eppendorf microtube without touching the tube wall, immediately frozen, and kept at −20°C until processed. A new patch pipette was used for each cell patch-clamped and harvested.

RT reaction
The RT reaction for each cell was tested on the harvested cytoplasm without prior RNA purification. Each individual cell cytoplasm was adjusted to 8.8μl with internal pipette solution and supplemented with 1.2μl random hexamer (50μM) oligonucleotides (Perkin Elmer), then heated to 65°C for 10 min. The RT reaction was carried out in a final volume of 20μl for each cell cytoplasm, under the following conditions: 50 mM Tris/HCl (pH 8.3), 75 mM KCl, 3 mM MgCl2, 0.5 mM each dATP, dCTP, dGTP, and dTTP (Perkin Elmer), together with 50 U MuLV RT and 20 U ribonuclease inhibitor (Perkin Elmer). The reaction mixture was incubated for 10 min at 20°C to hybridize the primers to the RNA. RT was carried out at 42°C for 30 min, followed by a 10 min incubation at 95°C, then chilled on ice and stored at −20°C until PCR amplification.

PCR amplification
PCR was carried out by adding 5μl RT template to a mixture of (final concentrations): 50 mM KCl, 10 mM Tris/HCl (pH 8.3), 4.5 mM MgCl2, 200 μM each dNTP, 1 μM each primer, and 1 U Amplitaq Gold in a final volume of 25μl. DNA conditions included an initial denaturation step of 10 min at 95°C, and 45 cycles (for β-actin) or 45+15 cycles (for TRPM8, AR, K5, K8, K14, and K18 mRNA) of 20 s at 95°C, 20 s at 60°C, and 30 s at 72°C, followed by 7 min at 72°C. The PCR products for all markers were analysed on agarose gel after 45 amplification cycles and an 15 additional PCR cycles were carried out to improve detection of PCR-amplified fragments. The 15 additional cycles needed for mRNA detection were carried out using 1 μl of the first PCR product as a template under the same reaction conditions as for the first PCR.

Controls
mRNA from LNCaP cells was used as a positive control for TRPM8 and AR mRNA detection. A pipette control (with the internal solution) was carried out under the same conditions as applied for harvesting the cell cytoplasm, to check for RNA or plasmid contamination by the lysed cells in the cell bath medium. Total RNA from hPCE cells was used as a control for all keratins.

Western-blot assay
AR-transfected PNT1A cells (control or siRNA-transfected) were harvested in PBS then sonicated in an ice-cold buffer (pH 7.4) containing 20 mM Hepes, 50 mM NaCl, 10 mM EDTA, 1 mM EGTA, 1 mM PMSF, 1% Nonidet P40, a mixture of protease inhibitors (Sigma, L’Isle d’Abeau Chesnes, France), and a phosphatase inhibitor (sodium orthovanadate; Sigma). Samples were electrophoretically analysed on 10% polyacrylamide gel using the SDS/PAGE technique. The proteins were then transferred for 1 h (50 mA, 25 V) onto a nitrocellulose membrane using a semi-dry electrobetter (BioRad). The membrane was cut into thin equal strips and processed for Western blot. The strips were first blocked in 5% TNT-milk (15 mM Tris buffer, pH 8, 140 mM NaCl, 0.05% Tween 20, and 5% non-fat dry milk) for 30 min at room temperature, washed in TNT (three times), then soaked in primary anti-AR (MS-443-PO; Neomarkers) or anti-actin (MS-1295-P; Neomarkers) antibody, diluted 1/300 and 1/500 respectively, in TNT-milk for 1 h at room temperature. After three washes in TNT, the strips were transferred into anti-mouse IgG horseradish peroxidase-linked secondary antibodies (Chemicon, Temecula, CA, USA), diluted in TNT-milk (1/20000) for 1 h. After three 10-min washes in TNT, the strips were processed for chemiluminescence detection using Supersignal West Pico chemiluminescent substrate (Pierce Chemical Company, Rockford, IL, USA) according to the manufacturer’s instructions. The blots were then exposed via free access.
to X-Omat AR films (Eastman Kodak Company, Rochester, NY, USA).

Immunohistochemistry studies
Resection specimens from human prostate were frozen in liquid nitrogen-cooled isopentane and kept in Tissue-Tek at −80°C. 10 μm sections were prepared at −20°C with a cryostat and mounted on glass slides. The sections were blocked with PBS/1.2% gelatine (PBS/gelatine) for 30 min at 37°C, then co-incubated with a rabbit anti-TRPM8 channel antibody (ab3243; Abcam; dilution, 1/100) and either mouse anti-cytokeratin 14 (anti-CK14) antibody (MAB3232; Chemicon; dilution, 1/200) or mouse anti-CK18 antibody (MS-919-S1; Neomarkers; dilution, 1/200) or mouse anti-α-smooth actin antibody (MAB1522; Chemicon; dilution, 1/200) diluted in PBS/gelatine for 1 h at 37°C. After thorough rinsing in PBS/gelatine, the slides were treated with the corresponding Alexa fluor 488-labeled anti-rabbit IgG (A-21206; Molecular Probes; dilution, 1/2000) or Rhodamine RedX-labeled anti-mouse IgG (715-295-151; Jackson Immunoresearch Laboratories; dilution, 1/100) diluted in PBS/gelatine for 1 h at room temperature. After rinsing twice in PBS/gelatine and once in PBS, the slides were mounted with Mowiol and examined under a confocal microscope (Zeiss LSM 510; acquisition parameters: objective 40×/1.4; thickness of confocal slide, 0.5 μm).

Data analysis
Each experiment was repeated at least three times and the results were expressed as means ± S.D. The data were analysed and graphs plotted using Origin 5.0 software (Microcal). InStat3 (GraphPad Software, San Diego, CA, USA) was used for statistical analysis and the mean values were compared using either unpaired t-test with Welch’s corrected test (two groups) or one-way ANOVA with Dunnett’s multiple comparison post-test (more than three groups).

Results
DHT is essential and sufficient to enhance trpm8 expression in a steroid-deprived LNCaP cell model
In a recent study Henshall et al. (2003) reported a loss of TRPM8 mRNA expression in a rat prostate cancer xenograft model after castration. In order to determine whether the human trpm8 gene was regulated by steroids (particularly androgens), we cultured TRPM8-expressing LNCaP cells under steroid-free conditions. Then, using semi-quantitative RT-PCR experiments, we studied the expression kinetics of TRPM8 mRNA in the LNCaP cells. As expected, the trpm8 gene was down-regulated under steroid deprivation. Indeed, as seen in Fig. 1A and B, the decrease in TRPM8 mRNA rate was detected 16 h after steroid deprivation of the cells and stagnated at a basal average level of 30 ± 8% of the control after 4 days. As DHT is known to be the main physiological steroid in the prostate, we studied the effects of adding DHT on trpm8 gene expression in LNCaP cells cultured under steroid-free conditions.

It has been shown previously that the androgen concentration differentially regulates gene expression in prostate carcinomas (Chang et al. 1997). We therefore started by examining the DHT dose-dependence of TRPM8 mRNA expression. LNCaP cells were cultured in a steroid-free medium for 6 days before incubation in a steroid-deprived medium supplemented with increasing concentrations of DHT (from 10⁻¹⁰ to 10⁻⁷ M) for 48 h. The LNCaP cells were harvested and analysed by semi-quantitative RT-PCR. As shown in Fig. 2A, the effect of DHT on trpm8 expression was concentration-dependent. At low physiological concentrations (10⁻¹⁰, 10⁻⁹ and 10⁻⁸ M DHT), an increase in TRPM8 mRNA rate was detected (473 ± 26%, 428 ± 39%, and 386 ± 29% of control, respectively), whereas a higher concentration (10⁻⁷ M DHT) induced only a 2.7-fold increase (276 ± 3% of control). These results suggest that trpm8 gene expression is maximal at physiological concentrations of testosterone.

Secondly, we studied the DHT time-response of TRPM8 mRNA expression in LNCaP cells. A physiological dose of 10⁻⁹ DHT was added to LNCaP cells cultured for 6 days in steroid-free medium and incubated for varying lengths of time. In these experiments, the TRPM8 mRNA rate was up-regulated by factors of 2.7 (270 ± 62%), 3.4 (344 ± 126%), and 4.2 (422 ± 93%) after 2, 4, and 8 h DHT treatment, respectively (Fig. 2B and C). After 48 h, 10⁻⁹ M DHT treatment was able to restore trpm8 gene expression to levels (495 ± 55%) comparable to those observed in cells kept in complete medium for 48 h (518 ± 91%). In conclusion, our results clearly showed that DHT is essential and sufficient to regulate trpm8 gene expression in the LNCaP cell line.

TRPM8 expression involves the functional AR in human prostate cells
Androgen-sensitive genes are known to be regulated by DHT-dependent activation of AR. As LNCaP cells express a functional AR, we studied the consequences of anti-androgen treatment on trpm8 gene expression.
Figure 1  Steroid removal triggers down-regulation of \textit{trpm8} expression in the LNCaP cell line. (A) Agarose gel showing the down-regulation kinetics of the TRPM8 transcripts in LNCaP cells in steroid-depleted medium. Cells were cultured in steroid-free medium for up to 6 days. After each incubation time in steroid-free medium, the total RNA was extracted from the samples and a semi-quantitative RT-PCR was carried out for \textit{trpm8} gene (503 bp) expression, as described in the Materials and methods section. \textit{\beta}-Actin RNA expression (210 bp) was used as an internal standard to control the rate of the RNA in each sample studied. (B) In semi-quantitative RT-PCR studies, the density of each band was determined and the rate of TRPM8 RNA expression was normalized to that of \textit{\beta}-actin (the control, CTL). The kinetics of the variation in \textit{trpm8} expression is presented as a function of the incubation time of the LNCaP cells in steroid-free medium. PCR products were analysed on 1.5% agarose gel containing 0.5 mg/ml ethidium bromide. A 1 kb DNA ladder (M; bp) was used as a DNA size marker. Each experiment was repeated three times in three independent cell cultures and representative experiments are presented. Each experiment was repeated three times in three independent cell cultures. A representative experiment is presented in (A) and the means ± s.d. of three independent experiments in (B).

Figure 2  DHT is essential and sufficient to enhance \textit{trpm8} expression in the LNCaP cell line. (A) Dose-effect of DHT-induced \textit{trpm8} expression. LNCaP cells were cultured in steroid-free medium for 6 days prior to incubation with either medium alone (CTL) or with various concentrations of DHT (from 10^{-10} to 10^{-7} M) for 48 h. RT-PCR experiments were then carried out on each sample of total RNA for expression of the \textit{trpm8} gene. \textit{\beta}-Actin mRNA expression was used to control the RNA rate in each sample. (B) Kinetics of the DHT (10^{-9} M)-induced \textit{trpm8} RNA expression in LNCaP cells previously cultured in steroid-free medium for 6 days. The last line of the agarose gel represents the expression rate of TRPM8 mRNA in LNCaP cells incubated in a steroid-free medium for 6 days prior to incubation in complete medium for 48 h (48 h + CM). (C) Semi-quantitative RT-PCR experiments showing the variation in DHT (10^{-9} M)-induced expression of the \textit{trpm8} gene. Experiments were carried out on each total RNA sample, as described in the Materials and methods section and in Fig. 1. The histogram shows the semi-quantified TRPM8 mRNA level in LNCaP cells cultured either with DHT (10^{-9} M) alone (shaded bars) or with complete medium (white bar). PCR products were analysed on 1.5% agarose gel containing 0.5 \mu g/ml ethidium bromide. A 1 kb DNA ladder (M; bp) was used as a DNA size marker. Each experiment was repeated four times in four independent cell cultures. A representative experiment is presented in (B) and the means ± s.d. of four independent experiments in (C) (*P \leq 0.05; **P \leq 0.01).
Bicalutamide induced a decrease in TRPM8 mRNA in LNCaP cells. (A) The effects of bicalutamide treatment (10 µM for 3 days) on trpm8 gene expression were studied in LNCaP cells using the RT-PCR technique. β-Actin mRNA expression was used to control (CTL) the mRNA rate in each sample used for the RT-PCR. A no-template control (H2O) was also run with the PCR samples, where the cDNA was replaced with water. (B) The semi-quantitative RT-PCRs for the expression of the trpm8 gene were carried out on each total RNA sample, as described in the Materials and methods section and in Fig. 1. Bars on the panel represent means ± S.D. (n = 3 independent experiments; *P ≤ 0.05).

The trpm8 gene promoter presents putative distal and proximal AR response elements (AREs)

To determine whether trpm8 gene was a primary androgen-responsive gene, AR-transfected PNT1A cells were incubated in the presence of CHX and ANM (CHX + ANM), two protein-synthesis inhibitors, the trpm8-expression studies were conducted as described by Zhu & Wang (1999) in their studies of androgen-dependent expression of calreticulin. Basically, the AR-transfected cells were incubated for 24 h in a serum-free medium to let them produce the AR protein without activating it (incubation in steroid-deprived medium). The cells were then incubated in a complete medium supplemented with CHX + ANM to suppress the translation of transcription factors induced by AR. Figure 5A shows that the first wave expression was also inhibited (Fig. 4A). We then used AR-transfected PNT1A cells to investigate the ability of the AR to re-induce trpm8 gene expression. As described in the Materials and methods section, we cultured either AR- or AR-L707R-transfected PNT1A cells (with the Leu-707→Arg substitution responsible for complete androgen insensitivity; Lumbroso et al. 1996) in a steroid-free medium for 24 h. The medium was then removed (J0) and replaced with either complete or steroid-free medium for cell culture. Cells were harvested kinetically prior to RT-PCR studies. As expected, the psG5-hARwt transfection induced a strong transcription of the trpm8 gene in under 6 h (Fig. 4B). A weak TRPM8 mRNA expression was also detected in the AR-transfected PNT1A cells cultured without steroids, as well as in cells transfected with ARL, certainly due to the basal activity of the AR. In addition, we obtained the same results for PSA mRNA detection, using it as a positive control for AR activity (Fig. 4B). We finally checked trpm8 induction by transfecting siRNA-AR in PNT1A cells transfected by ARpsg5 cultured under steroid-free conditions. 12 h after siRNA-AR transfection, PNT1A cells were cultured in complete medium for 36 h. TRPM8 mRNA expression decreased (14±7% of control) after siRNA-AR transfection (Fig. 4C). An immunoblot experiment confirmed that AR was drastically suppressed after siRNA-AR transfection, dropping to 15±7% of the control detected in the RT-PCR experiment (Fig. 4C) and 7% of the control detected in the Western-blot assay (Fig. 4D). Finally, to investigate a possible direct effect of the AR on the trpm8 gene promoter, we carried out an in silico study to check the putative action of AR on the trpm8 gene promoter.

In order to study the dependence of trpm8 expression on the presence of a functional AR, we used an ectopic AR-expression model. The human prostate normal transformed PNT1A cell line is a good model for the re-induction of androgen-regulated genes (Avances et al. 2001). It is important to note that the characterization of PNT1A cells has shown that this cell line has lost AR expression (Berthon et al. 1997, Mitchell et al. 2000). We studied trpm8 expression in this cell line and, as expected, we observed that trpm8 expression was also inhibited (Fig. 4A). We then used AR-transfected PNT1A cells to investigate the ability of the AR to re-induce trpm8 gene expression. As described in the Materials and methods section, we cultured either AR- or AR-L707R-transfected PNT1A cells (with the Leu-707→Arg substitution responsible for complete androgen insensitivity; Lumbroso et al. 1996) in a steroid-free medium for 24 h. The medium was then removed (J0) and replaced with either complete or steroid-free medium for cell culture. Cells were harvested kinetically prior to RT-PCR studies. As expected, the psG5-hARwt transfection induced a strong transcription of the trpm8 gene in under 6 h (Fig. 4B). A weak TRPM8 mRNA expression was also detected in the AR-transfected PNT1A cells cultured without steroids, as well as in cells transfected with ARL, certainly due to the basal activity of the AR. In addition, we obtained the same results for PSA mRNA detection, using it as a positive control for AR activity (Fig. 4B). We finally checked trpm8 induction by transfecting siRNA-AR in PNT1A cells transfected by ARpsg5 cultured under steroid-free conditions. 12 h after siRNA-AR transfection, PNT1A cells were cultured in complete medium for 36 h. TRPM8 mRNA expression decreased (14±7% of control) after siRNA-AR transfection (Fig. 4C). An immunoblot experiment confirmed that AR was drastically suppressed after siRNA-AR transfection, dropping to 15±7% of the control detected in the RT-PCR experiment (Fig. 4C) and 7% of the control detected in the Western-blot assay (Fig. 4D). Finally, to investigate a possible direct effect of the AR on the trpm8 gene promoter, we carried out an in silico study to check the putative action of AR on the trpm8 gene promoter.
of androgen-induced transcription factors, mainly with the AR, were sufficient to induce expression of TRPM8 mRNA. These data strongly indicated that trpm8 was a primary androgen-responsive gene.

It is now well established that the AR and the glucocorticoid receptor bind to the six-nucleotide half-site consensus sequence 5'-TGTTCT-3' (Roche et al. 1992, Schoenmakers et al. 2000). The active AR homodimer binds to a second, partially palindromic, half-site, located three nucleotides from the first sequence. The structure of the androgen-dependent gene promoter, e.g. PSA, usually includes ARE close to the transcriptional start site and other AREs located several kilobase pairs upstream within enhancers (Nantermet et al. 2004). According to a recent study, potential direct target genes of AR should be induced more than twice, 24 h after DHT administration to steroid-depleted LNCaP cells (Jia et al. 2003). Furthermore, at least two hypothetical AREs, conserved through three species, should be located in the 5 kb upstream from the first known transcribed nucleotide (Tsavaler et al. 2001a). As our results show a 4.5-fold induction of the trpm8 gene after DHT administration, we aligned the 5 kb upstream from the +1 transcribed nucleotide of the trpm8 gene from rats, mice, and human genomic DNA (contig accession numbers AC095563.7, AC087780.19, and AC005538 respectively). In order to check the relevance of the
contig sequences, we compared highly conserved trpm8 promoter sequences at positions −45, −70, and −95 (Fig. 5B). Afterwards, on the basis of one recent study about the consensus sequence of ARE (Nantermet et al. 2004), we used Gene runner 3.05 software (Hastings Software) to search for conserved AREs using the following matrix: RKNWCWNNNWGHNHW. We found four putative AREs, which have been relatively well-conserved through evolution (Fig. 5C). These hypothetical AREs are located at about −700 to −4200 nucleotides from the first known transcribed nucleotide upstream from the trpm8 gene.

Taken together, these results strongly argue that the trpm8 gene is a primary androgen-responsive gene. However, biophysical studies are required to check that the putative AREs are functional.

All these results demonstrated that the trpm8 gene is regulated by the AR in human prostate cells, although the conclusions are restricted to two cell lines. Nevertheless, we tested the relevance of our assumptions by...
studying the expression of the trpm8 and AR genes in normal, benign hyperplastic (BHP), and cancerous human prostates (collected from patients undergoing a prostatectomy). Finally, we also investigated the location of the TRPM8 channel in the human prostate.

**The trpm8 gene is under AR control in normal, hyperplastic, and cancerous human prostate apical epithelial cells**

To confirm AR regulation of trpm8 in a more physiological context, we studied the correlation between the expression of trpm8 and AR genes using the SCRT-PCR approach. To avoid cell selection under culture conditions, experiments were carried out as soon as possible after cell dissociation from different tissues (one normal prostate from a patient with bladder cancer, one benign hyperplasia of the prostate, and one cancerous prostate with a total PSA of 11 ng/ml). The cells were dispatched in two sets: set 1 (six normal cells, six hyperplasic cells, and seven cancerous cells) and set 2 (six normal cells, six hyperplasic cells, and six cancerous cells). TRPM8 mRNA was expressed in 73% (15/19 in set 1 and 11/18 in set 2) of normal, hyperplasic, and cancerous human prostate cells tested (Fig. 6A): note that no particular expression differences were observed depending on the oncogenic status of the cells. AR mRNA was detected in 80% of the cells (12/19 in set 1). The degree of correlation between trpm8 expression and AR expression was 0.676.
TRPM8 channel is expressed in both androgen-dependent human prostate apical epithelial and smooth muscle cells

It is well documented that both human prostate apical epithelial cells and smooth muscle cells express the AR (Pelletier et al. 2000, Yamashita 2004). However, the cells expressing the TRPM8 protein in the prostate had not yet been identified. Our SCRT-PCR results demonstrated that secretory epithelial cells expressed TRPM8 mRNA. Therefore we carried out an immunohistochemical experiment on cryosections from a cancerous human prostate (Gleason score, 6; 12 ng/ml PSA). Alexa 488-conjugated TRPM8 antibody was expressed in most of the prostate areas studied, although the highest TRPM8 protein expression was restricted to luminal epithelial cell surface membranes (Fig. 7A, E, and I). The apical marker CK18, Alexa 546-conjugated, was only detected in secretory epithelial cells (Fig. 7B) and, as expected, it was strongly co-localized with the TRPM8 channel (Fig. 7C and D). The basal marker CK14, Alexa 546-conjugated, was shown in the basal epithelial cells (Fig. 7F). In agreement with our previous data, no co-localization was observed between the TRPM8 channel and CK14 (Fig. 7G and H). Smooth muscle cells were stained with Alexa 546-conjugated α-smooth actin (Fig. 7J). As expected, a co-localization of the TRPM8 channel with α-smooth actin was visible (Fig. 7K and L).

Taken together these data demonstrate that the TRPM8 protein is mainly expressed in the androgen-dependent apical epithelial cells of the human prostate. Furthermore, weaker expression of the TRPM8 channel has also been detected in androgen-sensitive smooth muscle cells.

Discussion

In this study, we report two major findings likely to enhance our understanding of the physiological and pathological roles of the TRPM8, previously known as the cold-menthol receptor, in the prostate organ. Firstly, we demonstrate that trpm8 gene expression is determined by a functional AR and that trpm8 may be a primary androgen-responsive gene. Secondly, we show that the AR-regulated TRPM8 channel is expressed in both the smooth muscle and apical epithelial cells of the cancerous human prostate.

Prostate-specific expression of the trpm8 gene in androgen-dependent cells

Recently, the expression pattern of trpm8 has been studied in several tissues (Tsavaler et al. 2001) and has
been shown to be almost exclusively expressed in the male reproductive system. In fact, the prostate is apparently the only normal human tissue outside the nervous system to express TRPM8-related transcripts (Tsavaler et al. 2001, Peier et al. 2002, McKemy et al. 2002, Henshall et al. 2003). Moreover, remaining at moderate levels in normal prostate *trpm8* expression increases significantly in prostate cancer (Tsavaler et al.

Figure 7 The TRPM8 channel is expressed in both apical epithelial and smooth muscle cells in the human prostate. Confocal detection of immunohistochemical sections showing the location of the TRPM8 channel (A, E, I), CK18 (B), CK14 (F), and α-smooth muscle actin (J) in human prostate cancer. Co-localization was detected for (i) the TRPM8 protein and the apical epithelial marker CK18 in apical epithelial cells (C and D); and (ii) TRPM8 channel and smooth muscle marker α-actin (K and L). TRPM8 protein and the basal epithelial marker CK14 were found not to be co-localized (G and H). Panels D, H, and L are magnifications of the images directly above them.
2001, Kiessling et al. 2003). According to the latter, other human non-prostatic primary tumours of the breast, colon, lung, and skin also become highly enriched in TRPM8 mRNA, whereas it is virtually undetectable in the corresponding normal tissues (Tsavalier et al. 2001).

Androgen plays an important role in the progression of prostate cancer. Indeed, AR, when bound to DHT, directs the expression of genes controlling prostate hypertrophy and cancer development. In this study, we show that the AR is a major switch for trpm8 expression and requires a very short delay for up-regulation (about 8 h for maximal trpm8 induction). In addition, androgen induction of TRPM8 mRNA resists to protein synthesis inhibition in AR-transfected PNT1A cells, suggesting that the trpm8 gene promoter is activated directly by the DHT–AR complex. In view of the evolutionary conservation of the potential ARE in the 5 kb upstream from the first transcript nucleotide and the ‘switching role’ of the AR on trpm8 expression, we assume that the AR could regulate the trpm8 gene promoter directly in a ligand-dependent manner. However, further DNA-binding experiments are needed to confirm this hypothesis. Intriguingly, in a recent micro-array study aimed at identifying the genetic pathways activated by the AR during the induction of proliferation, the authors did not identify trpm8 as an androgen-responsive gene (Nantermet et al. 2004). This may be principally explained by the small amount of TRPM8 mRNA in the LNCaP cell line used in this study, only detectable after PCR amplification assays (Kiessling et al. 2003).

**Physiological role of the TRPM8 channel in the prostate epithelium**

It is currently assumed that the AR is expressed in both intermediate and mature secretory epithelial cells (van Bokhoven et al. 2003, van Leenders et al. 2003, Nantermet et al. 2004). In this study, we demonstrate that trpm8 is a target of AR, and that both trpm8 and AR are expressed in glandular prostate epithelial cells (K8- and K18-positive freshly cultured primary prostate cells). Interestingly, under differentiation, the PCE cells lost K8, K18 and AR and, consequently, TRPM8 mRNAs, but acquired K5 and K14 basal epithelial prostate cell markers. In addition, our immunohistochemical experiments confirmed that the TRPM8 channel was expressed in CK18-positive cells but was not present in the basal layer of the prostate acinus. Interestingly, it is well known that basal epithelial cells acquire the AR expression that induces activation of its target genes through differentiation. In these newly differentiated apical epithelial cells, the AR-responsive genes initially triggered proliferation, followed by terminal differentiation in secretory cells expressing the TRPM8 channel on their luminal side.

Recent studies have shown that the TRPM8 channel cloned from rodent dorsal root ganglion neurons is functional and responds to cold-menthol stimuli (McKemy et al. 2002, Peier et al. 2002).

Although the natural TRPM8 activators in the prostate have not yet been identified, it is assumed that this cationic Ca2+-permeable ion channel plays a functional role in Ca2+ homeostasis in prostate cells. Indeed, we have recently shown that alterations in Ca2+ homeostasis and calcium entry via cationic channels in the TRP channel family play an important role in the apoptosis, neuroendocrine differentiation, and proliferation of human cancer prostate cells (Thebault et al. 2003, Vandenberghe et al. 2002, 2003a, 2003b, Vanoverbergh et al. 2004). It is therefore clear that the TRPM8 channel may also be involved in these processes, especially those where AR plays a central role. Recent work by Zhang & Barritt (2004) suggested that TRPM8 was required for the survival of prostate cancer cells; however, as only the LNCaP cell line was used in this study, further experiments using primary cultured epithelial cells are required to clarify this TRPM8 function in normal and cancerous prostate cells.

Furthermore, taking into account that, on the one hand, trpm8 is strongly expressed on the luminal side of glandular prostate secretory epithelial cells and, on the other hand, secretion is known to be closely regulated by calcium, the TRPM8 channel probably also plays a role in normal prostate secretion. As it is known that prostate secretion from apical epithelial cells is essential for sperm fertility (Elzanaty et al. 2002), the TRPM8 channel may, indirectly, play a role in sperm motility. Moreover, it is currently known that puberty triggers deep modifications in the structure and functions of the normal prostate. Newly synthesized DHT is the main active factor and, therefore, all androgen-responsive genes may be induced at this time. We noted from the microarray studies (Nantermet et al. 2004) that other transduction elements involved in calcium homeostasis, such as inositol 1,4,5-triphosphate receptor-3, S100A, S100P, calnexin, calreticulin, and calcineurin B are also regulated by androgens. Considering this, we suggest that an androgen-sensitive calcium signature plays a crucial role in normal prostate physiology and deregulation of this signature may lead to the pathological state of the tissue.
Clinical implications for prostate cancer

To date, there are few prostate-specific markers available, nor are there any molecular markers for predicting prostate cancer relapses in patients treated by radical prostatectomy. Although it has recently been reported that the trpm8 gene is up-regulated in prostate cancer (Tsavaler et al. 2001, Fuessel et al. 2003, Kiessling et al. 2003), the way in which trpm8 expression may be involved in the molecular staging of prostate cancer remains unclear. Our results show that trpm8 expression is determined by the functional AR and that cell transition to the specific case of androgen-independence acquired due to loss of the AR induces a loss of TRPM8 expression. However, prostate cancers have mainly been reported to either overexpress AR or present increased AR activity. As suggested by our results, these cancers were expected to show an increase in trpm8 gene expression. Fuessel et al. (2003) suggested that quantifying TRPM8 mRNA from patient biopsies may be useful in predicting the androgen-independence of prostate cancer associated with relapses. Indeed, the loss of trpm8 expression may reveal either a high-tumour-grade cancer with undifferentiated cells, or a pure neuroendocrine cancer where AR is not expressed (Aumuller et al. 2001). Our study could be used as a basis for clarifying the loss of trpm8 expression in the prostate cancers of patients who had received androgen therapy 3 months before radical prostatectomy (Henshall et al. 2003). Indeed, the inhibition of AR activity by drugs such as bicalutamide down-regulates the trpm8 gene. These results highlight the false-negative results that may result from the use of an androgen-responsive gene as a prostate cancer marker.

In summary, we demonstrated that trpm8 gene expression in prostate cells is regulated by the AR. Furthermore, we showed that the AR-regulated TRPM8 channel is expressed in both smooth muscle and apical epithelial cells in the human prostate. These fundamental results provide new insights into the physiological and pathological roles of the TRPM8 ion channel in the prostate, previously known as the cold-menthol receptor.

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