Intermediate-Conductance $Ca^{2+}$-Activated $K^+$ Channel, $K_{Ca}$3.1, as a Novel Therapeutic Target for Benign Prostatic Hyperplasia

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Received April 7, 2011; accepted May 19, 2011

ABSTRACT

Recently, a new experimental stromal hyperplasia animal model corresponding to clinical benign prostatic hyperplasia (BPH) was established. The main objective of this study was to elucidate the roles of the intermediate-conductance $Ca^{2+}$-activated $K^+$ channel ($K_{Ca}$3.1) in the implanted urogenital sinus (UGS) of stromal hyperplasia BPH model rats. Using DNA microarray, real-time polymerase chain reaction, Western blot, and/or immunohistochemical analyses, we identified the expression of $K_{Ca}$3.1 and its transcriptional regulators in implanted UGS of BPH model rats and prostate needle-biopsy samples and surgical prostate specimens of BPH patients. We also examined the in vivo effects of a $K_{Ca}$3.1 blocker, 1-$[2$-chlorophenyl][diphenylmethyl]-1$H$-pyrazole (TRAM-34), on the proliferation index of implanted UGS by measurement of UGS weights and proliferating cell nuclear antigen immunostaining. $K_{Ca}$3.1 genes and proteins were highly expressed in implanted UGS rather than in the normal host prostate. In the implanted UGS, the gene expressions of two transcriptional regulators of $K_{Ca}$3.1, repressor element 1-silencing transcription factor and c-Jun, were significantly down- and up-regulated, and the regulations were correlated negatively or positively with $K_{Ca}$3.1 expression, respectively. Positive signals of $K_{Ca}$3.1 proteins were detected exclusively in stromal cells, whereas they were scarcely immunolocalized to basal cells of the epithelium in implanted UGS. In vivo treatment with TRAM-34 significantly suppressed the increase in implanted UGS weights compared with the decrease in stromal cell components. Moreover, significant levels of $K_{Ca}$3.1 expression were observed in human BPH samples. $K_{Ca}$3.1 blockers may be a novel treatment option for patients suffering from BPH.

Introduction

Benign prostatic hyperplasia (BPH) is noncancerous enlargement of the prostate gland with aging. BPH is demonstrated in 50% of men by the age of 60 years and is evident in up to 90% of men by 85 years (Berry et al., 1984). BPH causes increased resistance to urine flow through the urethra and sometimes kidney damage, bladder stones, and urinary tract infections and thereby affects the quality of life in one third of men older than 50 years (Djavan, 2003). $\alpha_1$-Adrenoceptor antagonists are used clinically to decrease the periurethral tone of prostatic smooth muscle (Andersson and Gratzke, 2007) and can regulate prostatic growth by inducing apoptosis of epithelial, stromal, and smooth muscle cells, but their long-term use can induce therapeutic tolerance (Kojima et al., 2009a,b). Antiandrogen therapy or surgery is used to resolve mechanical outlet obstructions (Pollard et al., 1989). BPH is a proliferative process of both stromal and epithelial elements of the prostate arising in the periurethral and transition zones of the gland and is hormonally dependent on testosterone and dihydrotestosterone production (Berry et al., 1984). The major component of clinical BPH specimens is stromal extracellular matrix, which is organized by smooth muscle, fibrous tissue element, and collagen (Shapiro et al., 2003). ABBREVIATIONS: BPH, benign prostate hyperplasia; $K_{Ca}$3.1, intermediate-conductance $Ca^{2+}$-activated $K^+$ channel; VSMC, vascular smooth muscle cell; REST, repressor element 1-silencing transcription factor; UGS, urogenital sinus; AP-1, activator protein-1; PCR, polymerase chain reaction; bp, base pair; $K_{Ca}$, $Ca^{2+}$-activated $K^+$ channel; $I_{KCa}$, intermediate-conductance $K_{Ca}$ channel; PCNA, proliferating cell nuclear antigen; TRAM-34, 1-$[2$-chlorophenyl][diphenylmethyl]-1$H$-pyrazole; $K_{Ca}$3.1, large-conductance $Ca^{2+}$-activated $K^+$ channel.

This work was supported by Grants-in Aid for Scientific Research from the Japan Society for the Promotion of Science [Grants 20300027, 21500098] to Y.I. and S.O., respectively; Takeda Science Foundation (to S.O.); and Salt Science Research Foundation [Grant 10C8] (to S.O.).

Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.

doi:10.1124/jpet.111.182782.

The online version of this article (available at http://jpet.aspetjournals.org) contains supplemental material.
1992); however, this feature is histologically different from the testosterone-induced rodent BPH model (Ishigooka et al., 1996), and this animal BPH model is not adequate to elucidate the pathological mechanisms of stromal hyperplasia. Mori et al. (2009) recently established a new experimental rodent model characterized by stromal hyperplasia. Urogenital sinus implants into the ventral prostate, “implanted UGS,” contain a much higher ratio of stroma than the age-matched ventral prostate and the testosterone-induced BPH model and are similar to those in men with BPH.

Ca\(^{2+}\)-activated K\(^+\) channel (K\(_{Ca}\)) can communicate directly from Ca\(^{2+}\) signal pathways to changes in membrane potential required for various cellular processes. In excitable cells, K\(_{Ca}\) activation contributes to the posthyperpolarization that follows an action potential and generally suppresses membrane excitability, whereas in nonexcitable cells hyperpolarization by K\(_{Ca}\) activation increases the driving force for Ca\(^{2+}\) entry, strongly associating with sustained Ca\(^{2+}\) influx (Stockker, 2004). Intermediate-conductance K\(_{Ca}\) (IK\(_{Ca}\)) is present in undifferentiated vascular and urinary bladder smooth muscles and participates in the control of cellular functions such as cell proliferation and maintenance of resting tone (Ohya et al., 2000; Köhler et al., 2003). IK\(_{Ca}\) has been molecularly identified as K\(_{Ca}\)-3.1 (Ishii et al., 1997) and is a possible molecular target for pharmacological intervention in restenosis, urinary incontinence, prostate cancer, and autoimmune diseases (Köhler et al., 2003; Wulff et al., 2007; Ohya et al., 2009). In addition, at least two transcriptional regulators of K\(_{Ca}\)-3.1 have been identified in vascular smooth muscle cells (VSMCs) and T lymphocytes: repressor element-1 silencing transcription factor (REST) and nuclear transcription factor activator protein-1 (AP-1) (Fos/Jun) (Ghanshani et al., 2000; Cheong et al., 2005).

The main objective of this study was to elucidate the possibility that IK\(_{Ca}\) can be a potential therapeutic target in human BPH using a stromal hyperplasia BPH model. Expressions of K\(_{Ca}\)-3.1 genes and proteins in implanted UGS of stromal hyperplasia BPH model rats were compared with those of the normal host prostate by DNA microarray, real-time polymerase chain reaction (PCR), Western blot, and immunohistochemical analyses. We also determined whether implanted UGS weights were suppressed by treatment with 1-(2-chlorophenyl)diphenylmethyl-1\(H\)-pyrazole (TRAM-34), a specific IK\(_{Ca}\) inhibitor. Similar examinations were performed using human prostate needle-biopsy samples and surgical prostate specimens of BPH patients.

### Materials and Methods

RNA Extraction, Reverse-Transcription PCR, and Real-Time PCR. Experimental model rats for BPH with stromal hyperplasia were prepared as reported previously (Mori et al., 2009). Implanted UGS and normal host prostate in stromal hyperplasia BPH model rats were dissected 3 weeks after implantation. All of the experiments were carried out in accordance with the guiding principles for the care and use of laboratory animals (the Science and International Affairs Bureau of the Japanese Ministry of Education, Science, Sports and Culture) and also with the approval of the ethics committee of Nagoya City University and Taiho Pharmaceutical Co. Total RNA from human normal prostate was purchased from BD Biosciences (San Jose, CA) and BioChain (Hayward, CA) (21–50 years old, three distinct lot number samples). Total RNA extraction and reverse transcription were performed as reported previously (Ohya et al., 2009). We enrolled seven patients with BPH 54 to 86 years old (mean age = 70.4 ± 4.5 years). Prostate needle-biopsy samples and surgical prostate specimens of BPH patients were obtained with informed consent from all of the patients before the study after explaining the purpose and methods. The study was approved by the ethics committee of Nagoya City University. BPH was diagnosed on the basis of the International Prostatic Symptom Score, the quality of life index, digital rectal examination, ultrasonography, uroflowmetry, and prostate needle biopsy. The resulting cDNA product was amplified with gene-specific primers, designed using Primer Express software (version 1.5; Applied Biosystems, Foster City, CA).

Quantitative real-time PCR performed with the use of SYBR Green chemistry on an ABI 7700 sequence detector system (Applied Biosystems) as reported previously (Ohya et al., 2009). The following PCR primers for rat clones were used for real-time PCR: K\(_{Ca}\)-3.1 (NM_023021, 837–937), 101 base pair (bp); REST (NM_031788, 367–2489), 123 bp; c-Fos (NM_022197, 897–1025), 129 bp; BosB (NM_001013146, 1075–1209), 125 bp; Fos-1 (NM_012953, 484–968), 121 bp; Fra-2 (NM_012954, 99–227), 129 bp; c-Jun (NM_023185, 1195–1317), 123 bp; JunB (NM_023183, 357–492), 136 bp; JunD (NM_138875, 1036–1160), 125 bp; β-actin (NM_031444, 419–519), 101 bp. The following PCR primers for human clones were used: K\(_{Ca}\)-3.1 (NM_002250, 172–293), amplicon = 122 bp; REST (NM_056512, 1415–1545), 131 bp; c-Fos (NM_005292, 533–658), 126 bp; BosB (NM_006732, 1075–1209), 135 bp; Fra-1 (X16707, 680–781), 101 bp; Fra-2 (X16706, 102–226), 125 bp; c-Jun (NM_002228, 1851–1990), 149 bp; JunB (NM_002229, 271–391), 121 bp; JunD (NM_005354, 1027–1134), 108 bp; β-actin (NM_001101, 411–511), amplipiece = 101 bp. Regression analyses of the mean values of three multiplex real-time PCRs for log\(_{10}\)-diluted cDNA were used to generate standard curves. Unknown quantities relative to the standard curve for a particular set of primers were calculated, yielding transcriptional quantification of gene products relative to the endogenous standard β-actin. For PCR cloning of full-length K\(_{Ca}\)-3.1, the following PCR primers were used: K\(_{Ca}\)-3.1 (NM_023021, 77–1459), 1383 bp. To confirm the nucleotide sequences, amplified PCR products and plasmid constructs were sequenced with an ABI PRISM 3100 genetic analyzer (Applied Biosystems).

**Genome-Wide DNA Microarray Analysis.** To determine the quantity and purity of the RNA samples, purified DNase-free RNA from normal host prostate and implanted UGS was analyzed on a ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE) and a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). We then used 12 Affymetrix (Santa Clara, CA) Rat Genome 230 2.0 arrays. These microarrays contain >31,000 genes. Labeling, hybridization (for 16 h), washing, and scanning of the microarray were performed by Bio Matrix Research (Chiba, Japan) following the manufacturer’s specifications (i.e., One-Cycle Target Labeling kit). The arrays were scanned on the GCS 3000 Affymetrix high-resolution scanner and analyzed using GeneChip Operating Software version 1.4 (Affymetrix) and GeneSpring version 7.3.1 (Agilent Technologies). Gene expression data were normalized by preparing “Per-Chip_Only” and were confirmed by Pearson correlation. Comparative analysis among expression profiles of two samples was carried out using GeneSpring software. Only gene expression levels with statistical significance (p < 0.05) were recorded as being “present” above background levels, and genes with expression levels below this statistical threshold were considered as “absent,” as reported previously (Kojima et al., 2009a).

**Western Blot Analysis.** The plasma membrane protein fraction was prepared from rat tissues as reported previously (Ohya et al., 2005), and the nuclear protein fraction was prepared using a ProteoJet Cytoplasmic and Nuclear Protein Extraction kit (Fermentas UAB, Vilnius, Lithuania). Equal amounts of proteins were subjected to SDS-polyacrylamide gel electrophoresis (10%). Blots were incubated with anti-K\(_{Ca}\)-3.1 (Alomone Labs, Jerusalem, Israel), anti-REST (Millipore Corporation, Billerica, MA), and anti-c-Jun.
antibodies (Cell Signaling Technology, Danvers, MA), and then incubated with anti-rabbit horseradish peroxidase-conjugated IgG (Millipore Corporation). An enhanced chemiluminescence detection system (GE Healthcare Japan, Tokyo, Japan) was used for detection of the bound antibody. The resulting images were analyzed by a LAS-1000 device (Fujifilm, Tokyo, Japan).

**Immunohistochemical Analysis.** The implanted UGS from BPH model rats (4 weeks after implantation) and surgical prostate specimens from BPH patients were fixed in 10% neutral buffered formalin, embedded in paraffin, and thin-sectioned at 2 μm (Mori et al., 2009). Tissue sections were deparaffinized in xylene followed by dehydration in graded alcoholic solutions and phosphate-buffered saline. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol for 30 min at 4°C followed by washing with phosphate-buffered saline. Three different anti-KCa3.1 antibodies [APC-064 (Alomone Labs), PAB7986 (Abnova, Taipei, Taiwan), and ab83740 (Abcam plc, Cambridge, UK)] were used for immunostaining. All of the sections were developed by the avidin-biotin complex method. The sections also were stained with hematoxylin and eosin staining and Masson’s trichrome. Microscopic images of each section were captured on a computer and analyzed with an image analysis system (Win ROOF; Tech-Jam, Osaka, Japan).

**Measurement of Cell Proliferation by Proliferating Cell Nuclear Antigen Immunohistochemistry.** To examine the proliferation of stromal and epithelial compartments of implanted UGS after treatment with TRAM-34 (Sigma-Aldrich, St. Louis, MO), the expression of proliferating cell nuclear antigen (PCNA) was analyzed as reported previously (Mori et al., 2009). After sectioning and staining, PCNA positive and negative cells were counted in 10 separate microscopic fields for each graft. The percentage of PCNA-positive cells of total epithelium cells and stromal cells was calculated as the PCNA labeling index.

**In Vivo Proliferation Assay.** To examine the effect of a selective IKCa blocker TRAM-34 (150, 300, 600, and 1200 μg/kg) on prostatic growth in BPH model rats, the in vivo proliferation assay was performed as reported previously (Kojima et al., 2009a). The compound was administered subcutaneously 7 to 21 days after UGS implantation (150, 300, 600, and 1200 μg/kg s.c.). Vehicle was given in the same way. On day 21, the implanted UGS were collected and weighed and then fixed in formalin and embedded in paraffin.

**Statistical Analysis.** Statistical significance between two groups or among multiple groups was evaluated using Welch’s t test or Tukey’s test after the F test or analysis of variance. Data are presented as the means ± S.E.M.

**Results**

**Gene and Protein Expression of KCa3.1 in Implanted UGS and Normal Host Prostate.** We first examined the expression of KCa3.1 transcripts in normal prostate and implanted UGS of stromal hyperplasia BPH model rats using DNA microarray and real-time PCR analyses. As shown in Fig. 1A, the expression of KCa3.1 transcripts in implanted UGS was much higher than that in normal host prostate (23-fold, probe ID 1368930_at).

Quantitative real-time PCR analysis revealed that expression of KCa3.1 transcripts in implanted UGS was approximately 6-fold higher than that in normal host prostate. The expression of KCa3.1 relative to β-actin was 0.0032 ± 0.0004 (n = 8) and 0.022 ± 0.003 (n = 8) in normal prostate and implanted UGS, respectively. No significant differences in cycle threshold values at 0.2 between two groups were found: 17.6 ± 0.2 (n = 8) and 17.6 ± 0.1 (n = 8) in normal host prostate and implanted UGS. We next cloned full-length KCa3.1 from the host prostate and implanted UGS. By repetitive PCR cloning using at least three independent cDNA products, no spliced variants were identified in both tissues.

The expression levels of KCa3.1 proteins in implanted UGS were verified by Western blot analysis. To confirm that equal amounts of protein (20 μg) were loaded in each lane, the blots were stained with 0.2% Ponceau S after transfer. As shown in Fig. 1C (top panel), the anti-KCa3.1 antibody recognized a single band at approximately 50 kDa, which is similar to the predicted molecular mass of the KCa3.1 protein. Densitometric analysis revealed that KCa3.1 expression levels were significantly higher in implanted UGS than those in normal host prostate. When expressing the calculated values in the normal host prostate group as 1.0, the expression of KCa3.1 protein was 7.9 ± 1.6 in the implanted UGS group (n = 4) (Fig. 1C, bottom panel). When anti-KCa3.1 antibody was preincubated with the excess antigen peptide against which the antibody was generated, a band of approximately 50 kDa specifically disappeared but nonspecific weak bands did not (data not shown). Furthermore, immunohistochemical examination showed that the expression of KCa3.1 proteins was rarely detected in normal rat ventral prostate (data not shown), whereas larger amounts of KCa3.1-posi-
Implanted UGS and Normal Host Prostate.

perplasia BPH.

UGS in stromal hyperplasia BPH model rats. A potent

shani et al., 2000). AP-1 is composed of dimeric complexes of

nuclear transcription factor AP-1 in T lymphocytes (Ghan-
goxide-treated groups. Daily administration of TRAM-34 for 2

mg/kg s.c.), and the weights of implanted UGS on day 22

7 to 21 days after UGS implantation (300, 600, and 1200

inhibition at a dose of 1200 g/kg (n = 10), p < 0.01) (Fig. 2).

To assess the in vivo effect of TRAM-34 on the proliferation

index in the stromal hyperplasia BPH model, implanted UGS

samples on day 22 were analyzed by PCNA immunostaining

(Mori et al., 2009). PCNA-positive cells were quantified by
counting brown-stained cells within the total number of cells in

10 randomly selected fields at 400× magnification (Fig. 3).

In the TRAM-34-treated group (600 and 1200 µg/kg s.c.),

qualitative macroscopic examination of PCNA-stained sec-
tions showed a substantial decrease in PCNA-positive cells in

the stromal components compared with epithelial cells,

whereas in the vehicle control no significant changes in the

ratio of stromal to epithelial components were detected (Fig.

3, A–C). Without affecting the quantification of PCNA stain-
ing in epithelial components (Fig. 3D, closed circles), that in

stromal components showed a significant decrease in PCNA-

positive cells at 600 (39.6 ± 1.7%, n = 8, p < 0.05) and 1200

(31.7 ± 3.0%, n = 8, p < 0.01) µg/kg, s.c. TRAM-34 compared

with those in the vehicle control (47.2 ± 2.2%, n = 7) (Fig. 3D,

open circles). These results suggest that pharmacological

blockade of KCa3.1 may be effective for human stromal hy-

perplasia BPH.

Expressions of KCa3.1 Transcriptional Regulators in

Implanted UGS and Normal Host Prostate. In VSMCs,

the gene encoding KCa3.1 is suppressed by REST (Cheong et

al., 2005). The expression of KCa3.1 also is regulated by

nuclear transcription factor AP-1 in T lymphocytes (Ghan-

shani et al., 2000). AP-1 is composed of dimeric complexes of

the Fos (c-Fos, FosB, Fra-1, and Fra-2) and Jun (c-Jun, JunB,

and JunD) members (Wisdom, 1999). We therefore deter-

mined the expression levels of REST and Fos/Jun members.

By real-time PCR analysis, significantly lower expression of

REST transcripts was found in implanted UGS than that in

the normal prostate (Fig. 4Aa). Expressions of REST relative
to β-actin were 0.021 ± 0.003 and 0.010 ± 0.003 in the

normal prostate and implanted UGS, respectively. In addi-
tion, significantly higher expressions of c-Jun were found in

implanted UGS than those in the normal prostate (Fig. 4Ae).

Expressions of c-Jun were 0.009 ± 0.001 (n = 8) and 0.018 ±

0.002 (n = 8) in normal prostate and implanted UGS, respec-
tively. No significant differences in the expressions of the

other AP-1 components were detected between two groups

(Fig. 4, Ab–d, f, and g). Expressions of Fra-1 transcripts were

<0.003 in both groups (data not shown).

The expression levels of REST and c-Jun proteins in the

nuclear protein fractions of normal prostate and implanted

UGS were verified by Western blot analysis. As shown in Fig.

4, Ba and b, anti-REST and anti-c-Jun antibodies recognized

single bands at approximately 130 and 40 kDa, which are

similar to the predicted molecular masses of REST and c-Jun

proteins, respectively. Densitometric analysis revealed that

REST and c-Jun expression levels were significantly lower and

higher in implanted UGS than those in normal host prostate,

respectively. When expressing the calculated values in the

normal host prostate group as 1.0, the expressions of

REST and c-Jun proteins were 0.35 ± 0.05 and 3.43 ± 0.38

in the implanted UGS group, respectively (n = 4) (Fig. 4Bc).
When anti-REST and anti-c-Jun antibodies were preincubated with the excess antigen peptides against which the antibodies were generated, respectively, bands of approximately 130 kDa (REST) and 40 kDa (c-Jun) specifically disappeared but non-specific weak signals did not (data not shown).

**Expressions of KCa3.1 and Its Transcriptional Regulators in Prostate from Human BPH Patients.** We next determined the expressions of KCa3.1, REST, and Fos/Jun member transcripts in prostate needle biopsies from BPH patients by real-time PCR analysis. The mean serum prostate-specific antigen was 12.3 ± 4.6 ng/ml in BPH patients (n = 7). Normal prostates showed low expression of KCa3.1, whereas seven BPH samples showed high expression of KCa3.1 (Fig. 5A). Expressions of REST transcripts were correlated inversely to those of KCa3.1 transcripts in both groups (Fig. 5A). Expressions of KCa3.1/REST transcripts were 0.005 ± 0.001/0.043 ± 0.004 (n = 3) and 0.026 ± 0.004/0.020 ± 0.003 (n = 7) in normal prostate and BPH samples, respectively (p < 0.01 for each) (Fig. 5A). We also determined the expression levels of Fos/Jun members (Fig. 5B). Significantly higher expressions of Fra-2 and c-Jun were found in BPH samples than those in normal prostate (Fig. 5B). Expressions of Fra-2/c-Jun transcripts were 0.018 ± 0.003/0.016 ± 0.002 (n = 3) and 0.040 ± 0.007/0.037 ± 0.008 (n = 7) in normal prostate and BPH samples (p < 0.05 for each). There were no significant differences in the expressions of c-Fos and JunD between two groups (p > 0.05) (Fig. 5B). Expressions of FosB, Fra-1, and JunB transcripts were <0.007 in both groups (data not shown).

We further performed immunohistochemical staining of KCa3.1 proteins using surgical prostate specimens (n = 12) from BPH patients. We enrolled 10 patients with BPH 61 to 81 years old (mean age = 73.0 ± 1.8 years), and the prostate volume was 166.3 ± 63.0 ml (n = 10). In the normal human prostate, the volume was 15.0 ± 3.6 ml (n = 3). We used three different anti-KCa3.1 antibodies from Alomone Labs, Abnova,
and Abcam. Strong background signals were observed with anti-KCa3.1 antibodies (1:100) from Alomone Labs (data not shown), whereas there were relatively weak background signals with the other antibodies: Abnova (1:25) and Abcam (1:100). We considered the double-positive samples for both KCa3.1 and REST (Fig. 4Aa and c-Jun (Fig. 4Ae) were plotted against that of KCa3.1 (arbitrary unit) (Fig. 1B) as KCa3.1 versus REST (Fig. 6B) and KCa3.1 versus c-Jun (Fig. 6C), respectively. The KCa3.1 expression showed a negative correlation with REST expression \((n = 8, R = -0.78, p = 0.021)\) (Fig. 6B) and a positive correlation with c-Jun expression \((n = 8, R = 0.84, p = 0.010)\) (Fig. 6C), respectively. Likewise, expressions of REST (Fig. 5A) and c-Jun (Fig. 5B) in prostate needle biopsies from BPH patients were plotted against KCa3.1 (arbitrary unit) (Fig. 5A) as KCa3.1 versus REST (Fig. 6D) and KCa3.1 versus c-Jun (Fig. 6E), respectively. The KCa3.1 expression showed a negative correlation with that of REST \((n = 7, R = -0.81, p = 0.026)\) (Fig. 6D) and a positive correlation with that of c-Jun \((n = 7, R = 0.95, p < 0.001)\) (Fig. 6E). These findings strongly support our assumption that the down-regulation of REST and up-regulation of c-Jun contribute to facilitate KCa3.1 expression in implanted UGS and prostate from BPH patients.

**Discussion**

The identification of novel targets for BPH therapy is of great interest. The main findings of the present study indicated: 1) high level expression of KCa3.1 in the stromal components of implanted UGS in a new experimental stromal hyperplasia BPH model recently established by Mori et al. (2009) and human BPH samples and 2) the inhibitory effect of a KCa3.1 blocker, TRAM-34, on the in vivo proliferation of implanted UGS in stromal components.

IKCa/KCa3.1 is a regulator of cell proliferation in undifferentiated smooth muscles, tumors, and lymphocytes and provides new therapeutic target for restenosis, cancer, and autoimmune disease (Köhler et al., 2003; Wulff et al., 2007; Obya et al., 2009). Up-regulation of AP-1 and down-regulation of REST are associated with up-regulation of KCa3.1 (Ghanshani et al., 2000; Cheong et al., 2005; Tharp et al., 2008; Park et al., 2011).

AP-1 regulates cellular processes such as differentiation, proliferation, and apoptosis in response to a variety of stimuli. Ricote et al. (2003) have shown that no immunoreaction to AP-1 is detected in the normal human prostate; however, immunoreaction to AP-1 is positive in BPH. In the present study, we showed a significant increase in the gene and protein expressions of c-Jun but not other AP-1 components (Fos, FosB, Fra-1, Fra-2, JunB, and JunD) in implanted UGS compared with those in the normal rat prostate (Fig. 4). Likewise, up-regulation of KCa3.1 and c-Jun were observed in implanted UGS (Fig. 5B). Li et al. (2007) have shown that c-Jun is abundantly expressed in the prostatic stroma but not in the epithelium of human BPH samples, and c-Jun is expressed at high levels in BPH nodules enriched with stroma. In the present study, positive correlations between KCa3.1 and c-Jun were significant in both implanted UGS and prostate needle biopsies from BPH patients (Figs. 4Ae, 5B, and 6, C and E). These strongly suggest that the increased expression of KCa3.1 mediating the up-regulation of c-Jun may contribute to the promotion of proliferation in prostatic cells in the stromal region. In the present study, Fra-2 also was up-regulated significantly in human BPH

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**Fig. 5. Expression of KCa3.1, REST, and AP-1 components in human BPH samples.** A, real-time PCR analysis of KCa3.1 and REST in human normal prostate \((n = 3, \text{open columns})\) and BPH samples \((n = 7, \text{closed columns})\). Values are shown for steady-state transcripts relative to β-actin in the same preparation. Results are expressed as the means ± S.E.M. **B**, real-time PCR analysis of AP-1 components transcripts (c-Fos, Fra-2, c-Jun, and JunD) in human normal prostate \((n = 3, \text{open columns})\) and BPH samples \((n = 7, \text{closed columns})\). **C**, immunohistochemical analysis of KCa3.1 expression in human BPH samples. KCa3.1-positive, brown-stained signals, which are specific for anti-KCa3.1 antibody from Abnova, are detected in stromal but not in epithelial compartments. Original magnifications, 40×.
samples (Fig. 5B). A recent report has shown that Fra-2 contributes to prostate cancer cell growth (Kajanne et al., 2009), suggesting that the Fra-2 signal pathway may be responsible for the cell proliferation of prostatic cells in epithelial region components but not in stromal regions. Down-regulation of REST can be correlated with the up-regulation of KCa3.1 in implanted UGS of stromal hyperplasia BPH model rats (Fig. 4, Aa, Ba, and Bc). Negative correlations between KCa3.1 and REST were significant in both implanted UGS and prostate needle biopsies from BPH patients (Figs. 4Aa, 5A, 6, B and D). The expression of REST declines in undifferentiated VSMCs, showing an inverse relationship with functional KCa3.1 (Cheong et al., 2005). Neylon et al. (1999) have shown that smooth muscle cells exhibiting contractile function express large-conductance Ca2+-activated K+ channel (BKCa/KCa1.1) predominantly, whereas proliferative smooth muscle cells express IKCa/KCa3.1 predominantly. In addition to the up-regulation of KCa3.1 and down-regulation of REST, the DNA microarray assay indicated the significant down-regulation of KCa1.1 in the implanted UGS in the stromal hyperplasia BPH model (Supplemental Fig. S1). Stromal hyperplasia BPH is characterized by stromal reorganization due to the transdifferentiation of fibroblasts to myofibroblasts and smooth muscle cells (Bartsch et al., 1979). This suggests that the transcriptional regulation of KCa3.1 may be involved in stromal reorganization in stromal hyperplasia BPH. Taken together, similar to the recent reports showing correlation between KCa3.1, REST, and c-Jun expressions (Tharp et al., 2008; Park et al., 2011), concomitant up-regulation of AP-1 and down-regulation of REST can be suggested as a possible mechanism underlying the up-regulation of KCa3.1 in implanted UGS of stromal hyperplasia BPH model rats and may be responsible for the progressive growth of stromal compartments of BPH.

A selective KCa3.1 blocker, TRAM-34, reduces atherosclerosis development by the inhibition of both VSMC proliferation and T lymphocyte and macrophage activity (Wulff et al., 2007). The present study clarified the inhibitory effects of TRAM-34 on the in vivo proliferation of implanted UGS of
the stromal hyperplasia BPH model in a dose-dependent manner (Fig. 2). Moreover, in the TRAM-34-treated group, qualitative macroscopic examination of PCNA-stained sections showed a correlative decrease in PCNA-positive cells in stromal compartments including smooth muscle cells without any changes in those in epithelial compartments (Fig. 3). Recent studies have shown that KCa such as KCa3.1 and small-conductance Ca$^{2+}$-activated K$^+$ channel (SKCa$^{2+}$/KCa$^{2+}$, 2.2) participate in the modulation of cell proliferation in several types of cells and that their pharmacological blockade and down-regulation inhibit cell proliferation by G$\text{0/G1}$ arrest (Morimoto et al., 2007; Wang et al., 2007; Grgcic et al., 2009). This suggests that the inhibitory effects of TRAM-34 on in vivo cell proliferation in the implanted UGS of stromal BPH model rats may be responsible for the termination of cell cycle progression from G$\text{0/G1}$ by TRAM-34 treatment.

Although $\alpha_1$-adrenoceptor antagonists are considered an appropriate treatment for all BPH patients, irrespective of prostate size, combination therapy with $\alpha_1$-adrenoceptor antagonists and 5$\alpha$-reductase inhibitors is a recently accepted treatment for patients with clinically enlarged prostates (McConnell et al., 2003). 5$\alpha$-Reductase inhibitors are well tolerated, but the most common adverse effects are sexual dysfunction, including a reduced libido, erectile dysfunction, and, less frequently, ejaculation disorders (Gravas and Oelke, 2010); therefore, other options are required to reduce prostate volume and the risk of clinical progression of BPH. Daily administration of TRAM-34 at relatively high concentration did not induce any changes in blood chemistry or hematology or at necropsy of the major organs of rodents (Si et al., 2006). Pharmacological blockade of KCa3.1 may be relatively safe and well tolerated as a therapeutic target of BPH. In addition, pharmacological blockade of KCa3.1 prevents up-regulation of KCa3.1 and down-regulation of REST in coronary arteries of the postangioplasty restenosis model (Tharp et al., 2008). This suggests that pharmacological blockade of KCa3.1 prevents phenotypic modulation of prostatic stromal cells in stromal hyperplasia BPH. Moreover, Tharp et al. (2006, 2008) have shown that in proliferative, dedifferentiated VSMCs cells up-regulation of KCa3.1 is followed by KCa3.1 promoter histone acetylation. This suggests that TRAM-34 also prevents KCa3.1 promoter activation at the epigenetic level. Further study will provide more detailed mechanisms underlying TRAM-34-induced anti-proliferative effects in prostatic stromal cells from BPH patients.

In VSMCs with a dedifferentiated, proliferating phenotype, hyperpolarization by K$^+$ channel activation increases the driving force for Ca$^{2+}$ entry via voltage-independent Ca$^{2+}$ channels such as store-operated Ca$^{2+}$ channel, receptor-operated Ca$^{2+}$ channel and Ca$^{2+}$-release activating Ca$^{2+}$ channel, which are encoded by mammalian homologues of transient receptor potential genes and Orai/stromal interaction module genes (Inoue et al., 2006; Albert et al., 2007; Guibert et al., 2008; House et al., 2008). However, molecular components directly involving VSMC proliferation remain to be determined. Dedifferentiated VSMCs are characterized by loss of voltage-gated Ca$^{2+}$ channel, which is a major Ca$^{2+}$ channel component in smooth muscle cells with a differentiated, contractile phenotype. In implanted UGS of stromal hyperplasia BPH model rats, voltage-gated Ca$^{2+}$ channel was rarely expressed (data not shown). In the present study, the molecular identification and functional characterization of voltage-independent Ca$^{2+}$ channels remain to be determined in either implanted UGS or prostatic tissues from BPH patients. Several researchers have reported that down-regulation of KCa3.1 and voltage-gated K$^+$ channel is observed in lower urinary tracts in BPH and prostatitis patients (Liang et al., 2006; Chang et al., 2010). DNA microarray analysis showed that KCa3.1 transcript expression (probe ID 1372929_at) in implanted UGS was markedly lower than that in the normal host prostate. No significant difference in voltage-gated K$^+$ channel transcript expression (probe ID 1369802_at) was detected between them (Supplemental Fig. S1); however, the relative expression level of voltage-gated K$^+$ channel was much lower than that of KCa3.1 in implanted UGS and prostate from BPH patients (data not shown). The possibilities of voltage-independent Ca$^{2+}$ channels and other K$^+$ channel subtypes as therapeutic targets for BPH are also pressing questions.

In summary, our present study suggests that the activation of Ca$^{2+}$-release activating Ca$^{2+}$ channels by up-regulation of KCa3.1 may be implicated in the development of BPH, which represents a field of great interest for scientific and clinical research communities. We further showed that both c-Jun and REST are candidates for the transcriptional regulation of KCa3.1 in stromal compartments of BPH. In vivo treatment with TRAM-34 significantly suppressed cell proliferation in stromal compartments, supporting the notion that KCa3.1 blockers may be a novel treatment option for patients suffering from stromal hyperplasia BPH.

Acknowledgments
We thank to Dr. Oda (Taiho Pharmaceutical Co., Ltd., Japan) for giving us valuable advice and suggestions.

Authorship Contributions
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Supplemental Data

Intermediate-conductance Ca$^{2+}$-activated K$^+$ channel, $K_{Ca}3.1$, as a novel therapeutic target of benign prostatic hyperplasia

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*J Pharmacol Exp Ther*
Supplemental Figure S1. K_{Ca}1.1 and K_{V}1.3 transcript expressions in normal prostate and implanted UGS of stromal hyperplasia BPH model rats. A, B: DNA microarray analysis of K_{Ca}1.1 (A) and K_{V}1.3 (B) gene expressions. Expression levels were shown as normalized amounts taking the values in normal host prostate as 1.0.