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# Functional Role of PKC in Contraction of Cultured Human Prostatic Stromal Cells

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**Abstract** The contractile activity of prostatic stromal cells contributes to symptoms of benign prostatic hyperplasia (BPH). However, the mechanisms for this contraction have not yet been fully elucidated. In this study, we investigated the role of protein kinase C (PKC) in prostatic contraction by measuring the isometric tension development of cultured human prostatic stromal cells (CHPSCs) derived from BPH patients. Fresh human BPH tissue was used only in a Western blot analysis. A ring preparation made of CHPSCs and collagen gel could develop an isometric tension during activation with various agonists. Phorbol 12,13 dibutyrate (PDBu), a PKC activator, induced a relaxation. A Western blot analysis revealed the expression of PKC-potentiated protein phosphatase-1 inhibitory protein (CPI-17) in both CHPSCs and fresh human BPH tissue to be much lower than that in the rabbit aorta. When CPI-17 was over-expressed, PDBu induced a large contraction, but the agonist-induced contraction did not become larger than expected. In α-toxin permeabilized preparations, PDBu induced a relaxation in control CHPSCs, while it induced a contraction at a constant $[Ca^{2+}]_i$ in CPI-17 over-expressing CHPSCs. These results indicated that the activation of PKC in CHPSCs induces a relaxation probably due to low expression level of CPI-17 and also that the PKC-CPI-17 pathway does not appear to play a major role in the agonist-induced contraction even when CPI-17 was over-expressed. J. Cell. Biochem. 96: 65–78, 2005. 

**Key words:** PKC; CPI-17; prostate

Benign prostatic hyperplasia (BPH) is one of the most common diseases in elderly men and it is characterized by an abnormal proliferation of prostatic stromal and epithelial cells [Berry et al., 1984]. An excessive growth of the prostate compresses the prostatic urethra, which thus results in BPH symptoms such as dysuria, nocturia, and urgency. On the other hand, the contractile activity of prostatic stromal cells (smooth muscle cells and fibroblasts) also contributes to BPH symptoms and this is considered to be another major factor [Caine, 1988]. The contractile properties in response to various receptor stimulations have been well characterized and the involvement of $\alpha_1$-adrenoceptors [Caine et al., 1975; Hedlund et al., 1985], endothelin receptors [Langenstroer et al., 1993; Saita et al., 1997], 5-HT receptors [Horby-Petersen et al., 1985; Killam et al., 1995], and tachykinin $NK_2$ receptors [Palea et al., 1996] in the prostatic contraction have been documented. Among these receptors, $\alpha_1$-adrenoceptors are considered to be dominant and their antagonists have been successfully used to relieve BPH

Abbreviations used: BPH, benign prostatic hyperplasia; CHPSCs, cultured human prostatic stromal cells; CPI-17, PKC-potentiated protein phosphatase-1 inhibitory protein; DMEM, Dulbecco's modified Eagle's medium; EGTA, ethylene glycol-bis (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; eYFP, enhanced yellow fluorescent protein; FBS, fetal bovine serum; GTP, guanosine-5′-triphosphate; MLC, myosin light chain; MLCK, myosin light chain kinase; MLCP, myosin light chain phosphatase; PBS, phosphate-buffered saline; PDBu, phorbol 12,13 dibutyrate; PKC, protein kinase C; PSS, physiological saline solution; TURP, transurethral resection of the prostate.

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symptoms [Thiyagarajan, 2002]. However, little information is still available regarding the intracellular mechanism of prostatic contraction, although the involvement of GTP-binding proteins [Eckert et al., 1995; Saita et al., 1997] and $Ca^{2+}$ mobilization via voltage-dependent $Ca^{2+}$ channels and intracellular $Ca^{2+}$ release [Eckert et al., 1995; Saita et al., 1997] has been reported.

Protein kinase C (PKC) is one of the major molecules that are involved in the contractile pathway in smooth muscle. [Rasmussen et al., 1984; Nishimura et al., 1989; Masuo et al., 1994]. Recent studies have demonstrated that PKC-mediated phosphorylation of PKC-potentiated protein phosphatase-1 inhibitory protein (CPI-17) at Thr-38 induced the inhibition of myosin light chain phosphatase (MLCP), which resulted in an increase in MLC phosphorylation and tension at a constant $[Ca^{2+}]_i$ [Eto at al., 1997]. In contrast, however, a PKC-induced relaxing effect has also been reported. The application of PKC enzyme into detergent-skinned fiber was demonstrated to induce a relaxation [Inagaki et al., 1987; Parente et al., 1992]. In addition, PKC-activation has also been reported to induce a relaxation of the intact rat uterus [Baraban et al., 1985; Kim et al., 1996], guinea-pig ileum [Baraban et al., 1985; Menkes et al., 1986], and guinea-pig trachea [Menkes et al., 1986]. In the human prostate, the contractile effect of PKC-activation assessed by a reduction of cell length in a single cell study [Cook et al., 2002; Haynes et al., 2002] has been reported, while Marshall et al. [1999] suggested that PKC inhibitor did not alter the contraction to noradrenaline and human prostatic tissue showed no contraction after the administration of phorbol 12,13 dibutyrate (PDBu), a PKC activator. Accordingly, it can be said that the functional role of PKC in human prostate remains equivocal [Eckert et al., 1995].

In functional experiments of the prostate, it is very valuable to use human tissue specimens. In previous studies, human tissues were usually obtained from BPH patients undergoing transurethral resection of the prostate (TURP) or open prostatectomy [Ishigooka et al., 2000; Takahashi et al., 2003]. However, until now, there have been relatively few reports in which the intracellular mechanism for the contraction of human prostatic tissue has been investigated, probably due to the difficulty of obtaining sufficient amounts of fresh human BPH tissue. Recently, several papers have reported that cultured human prostatic stromal cells (CHPSCs) could be obtained from surgical prostate specimens by combined mechanical and enzymatic dissociation [Kassen et al., 1996; Jansen et al., 2000]. In addition, Kolodney and Wysolmerski [1992] reported a method to measure the isometric tension development of cultured cells using collagen gel. In the present study, we applied the above-mentioned collagen gel technique to CHPSCs and established a method to measure the isometric tension development of CHPSCs. Furthermore, we also established a method to achieve a high efficiency gene transfer into these cells. Using these methods, the functional role of PKC in contraction of CHPSCs was examined.

## MATERIALS AND METHODS

### Cell Culture

Human BPH tissue specimens were obtained from two patients subjected to TURP due to symptomatic BPH. Informed consent for the use of these specimens for the experiments was obtained from all patients well in advance of surgery. In addition, these tissue specimens were analyzed by the pathologist and diagnosed as nodular hyperplasia, which consist of hyperplastic prostatic glands and fibromuscular stroma. The surgical specimens were minced into small pieces and incubated for 1 h in Dulbecco's modified Eagle's medium (DMEM) (Sigma Chemical Company, St. Louis, MO) containing 1 mg/ml collagenase type I (Worthington, Lakewood, NJ) and 10 U/ml elastase (Wako, Osaka, Japan). After collagenase plus elastase digestion, the cells were washed and mixed with 2.4 mg/ml type I collagen (Cell matrix type IP, Nitta gelatin, Osaka, Japan) and then placed on a plastic dish, which was placed in a $CO_2$ incubator kept at 37°C for 1 h until collagen gel became solid and filled with DMEM. The cells that then grew out from the collagen gel were harvested and maintained in DMEM supplemented with 10% FBS, 100 μg/ml streptomycin, 100 units/ml penicillin, and 50 μg/ml ascorbic acid, which is reported to increase the contractility of vascular smooth muscle [L'Heureux et al., 2001]. Cells from passages 6–10 were used in this study.

### Immunofluorescence

The cultured cells were fixed with 2% formaldehyde for 20 min. Next, the cells were washed with phosphate-buffered saline (PBS) and permeabilized with 0.1% Triton X-100 for 5 min and incubated with 5% bovine serum albumin for 60 min. After washing with PBS, the cells were incubated with anti-vimentin antibody (1:100, Sigma Chemical Company) or anti-α-smooth muscle actin antibody (1:100, Sigma Chemical Company) for 60 min at room temperature. After washing with PBS, secondary antibody (1:100, Sigma Chemical Company) was added for 60 min. After washing with PBS, Hoechst 33258 (5 μg/ml, Sigma Chemical Company) was added for 5 min and then the cells were covered with glycerol-containing PBS (1:1) and visualized by a fluorescent microscope.

### Production of a Reconstituted Ring Preparation and Tension Measurement

The cells were collected by trypsinization and mixed with a culture medium ($5 \times 10^6$ cells/ml) containing 0.48 mg/ml type I collagen (Nitta gelatin). One hundred μl of the mixture containing $5 \times 10^5$ cells was placed around the $\phi = 2$ mm silicone column, which was attached on the center of the $\phi = 8$ mm silicone disc. After the collagen gel became solid, a culture medium was added and placed in a $CO_2$ incubator kept at 37°C for 12–24 h. During this period, the collagen gel shrank to form a ring preparation because of the interaction between cells and collagen gel matrix. The ring preparation so obtained was mounted between two tungsten wires, one of which was fixed and the other one was attached to a force transducer (UL2; Minebea Co., Nagano, Japan). These strips were placed in a physiological saline solution (PSS) consisting of the following composition (mM): NaCl, 123; KCl, 4.7; $CaCl_2$, 1.25; $MgCl_2$, 1.2; $KH_2PO_4$, 1.2; $NaHCO_3$, 15.5; D-glucose 11.5; pH 7.4. High-$K^+$ PSS was made by the equimolar substitution of KCl for NaCl. All tension experiments were performed at room temperature.

### Western Blot Analysis

Cultured cells were collected by trypsinization and placed in an extraction buffer (50 mM Tris-HCl (pH = 7.2), 1% TritonX-100, 0.5% Sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 10 mM $MgCl_2$, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μM 4-aminidophenylmethane sulfonyl fluoride). Regarding the tissue samples, they were freeze-fractured and also placed in the above mentioned extraction buffer. Protein extract was separated by SDS–PAGE and transferred to PVDF membrane. The membrane was subjected to immunoblotting with anti-CPI-17 (1:2,000, Upstate, Waltham, MA) antibody or anti-RhoA antibody (1:100, Santa Cruz Biotechnology, Santa Cruz, CA) and the signals were visualized using an enhanced chemiluminescence (ECL) detection system (Amersham Biosciences, Buckinghamshire, England). The transferred membrane was finally stained with Coomassie brilliant blue and the bands corresponding to actin were evaluated.

### Isolation of CPI-17 cDNA by RT-PCR

For the isolation of pig CPI-17 cDNA, RT-PCR was performed using total RNA obtained from pig aorta as a template. RT-PCR was done as previously reported [Nishimura et al., 1992]. The sequences of the forward, reverse and RT primers for pig CPI-17 were 5′-Agg ggT ACC ATg gCA gCT CAg Cgg CTg gg-3′, 5′-ggg gTA CCA gAg gCC ggg gCA TgC gTC Ag-3′ and 5′-AgC Cgg gCC gTg TTg Agg-3′, respectively, according to the published sequence reported by Eto et al. [1997]. For the introduction of mutation (CPI-17 T38A), PCR was used. All the PCR product inserted into the plasmid were sequenced using ABI Prism 310 Genetic Analyzer (Perkin-Elmer, Foster City, CA). The obtained cDNA and mutants were digested by Kpn I and subcloned into the baculovirus transfer vector described below.

### Vector Construction

Baculovirus vector containing a CMV promoter, which can be used for the efficient gene transfer into mammalian cells, was constructed according to the methods described by Shoji et al. [1997]. A mammalian expression vector, pEYFP-C1 (BD Biosciences Clontech, Tokyo, Japan), was digested by restriction enzymes, Apa LI and Afl II. The fragment including CMV promoter, eYFP (enhanced yellow fluorescent protein), MCS and SV40 poly A signal was taken and blunt-ended by KOD taq polymerase (TOYOBO, Osaka, Japan). This fragment was inserted into the MCS of a baculovirus transfer vector, pFastBac HTa (Life Tech). We named this new vector as pBYM (Baculovirus transfer vector for eYFP fusion protein in Mammalian

cell) vector. The Kpn I-digested cDNA (CPI-17 or CPI-17 T38A,) was subcloned into the pBYM vector at the MCS originally derived from pEYFP-C1 plasmid.

### Generation of Recombinant Baculovirus and Purification

The recombinant baculovirus was generated using a transfer vector (pBYM alone, pBYM+CPI-17, and pBYM+CPI-17 T38A?) and Bac to Bac system (Life Tech), according to the manufacturer's instructions. Recombinant baculoviruses were grown in *Spodoptera frugiperda* (Sf) 9 cells in Sf-900 II SFM supplemented with 1% fetal bovine serum (FBS), 100 mg/ml streptomycin, and 100 U/ml penicillin. For the purification of recombinant baculovirus, Sf9 cells were grown in 200 ml of culture medium in a 500 ml bottle set in a rotating shaker at 130 rpm at 28°C. When the culture reached around $10^6$ cells/ml, cells were infected by each recombinant baculovirus at MOI around 10. The culture supernatant was harvested by 10 min centrifugation at 1,000*g* for 10 min, 3–6 days after infection, depending on the morphological changes of the infected cells. Virus particles were pelleted by ultracentrifugation at 20,000 rpm in a Beckman 45 Ti angle rotor. The resultant pellet was resuspended in 6 ml PBS, vortexed vigorously, and centrifuged again at 2,800*g* for 20 min to remove the cell debris and aggregated virus. The final supernatant was filtered through the 0.45 μm filter and this filtrate was used for the transfection of CHPSCs.

### Transfection

When CHPSCs grown in a culture dish ($\phi =$ 6 cm) reached almost confluent, recombinant baculovirus was added at MOI around 100. After the addition of virus, cells were placed in a $CO_2$ incubator kept at 30°C. After 54 h, the virus-containing medium was removed, and the culture dish was inverted and the cells were observed by a fluorescent microscope from the bottom of the dish, so that we can confirm the expression of the eYFP-tagged protein in the living cells without contamination.

### Tension Measurement of α-Toxin Permeabilized Rings

Permeabilization of the CHPSCs rings was carried out using α-toxin according to the method described by Nishimura et al. [1988]. The rings were permeabilized in a relaxing solution (mM): (potassium methansulphonate 100, $Na_2ATP$ 2.2, $MgCl_2$ 3.38, ethylene glycol-bis (β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) 10, creatine phosphate 10, Tris-maleate 20 (pH = 6.8)) containing 5,000 U/ml *Staphylococcus aureus* α-toxin for 10–20 min. To deplete $Ca^{2+}$ stores of sarcoplasmic reticulum, every strip was treated with 10 μM A23187 (Calbiochem, La Jolla, CA) for 30 min in the relaxing solution [Kitazawa et al., 1991]. The activating solutions containing the indicated concentrations of free $Ca^{2+}$ were made by adding an appropriate amount of $CaCl_2$ to the relaxing solution, using a $Ca^{2+}$–EGTA binding constant of $10^6$/M [Saida and Nonomura, 1978]. These experiments were all performed at room temperature.

### Statistical Analysis

All data were expressed as the mean ± standard error of the mean (SEM) along with the number of observations (n). Unpaired Student's *t*-test was used to determine the statistical differences between the two mean values. A value of $P < 0.05$ was considered to be significant. All data were collected using a computerized data acquisition system (PowerLab; Analog Digital Instruments, Australia, and Macintosh Apple Computer).

## RESULTS

### Characterization and Contractile Activity of Cultured Human Prostatic Stromal Cells (CHPSCs)

Double-stain with vimentin (Fig. 1A) and Hoechst 33258 (Fig. 1B) was performed on CHPSCs. In order to visualize all cells, the cell nuclei were stained with a DNA staining dye, Hoechst 33258. As shown in Figure 1A and B, almost all cultured cells exhibited positive immunofluorescence staining for vimentin. Next, in order to determine to what extent the smooth muscle cells exist, double-stain with α-smooth muscle actin (Fig. 1C) and Hoechst 33258 (Fig. 1D) was performed. The percentage of smooth muscle cells was determined in 12 different fields of vision and 90 ± 1% of the cells was positively stained with α-smooth muscle actin antibody (Fig. 1C–E). The reconstituted ring preparations made of these cells produced an isometric tension development in response to endothelin-1 (ET-1) or U46619, a thromboxane

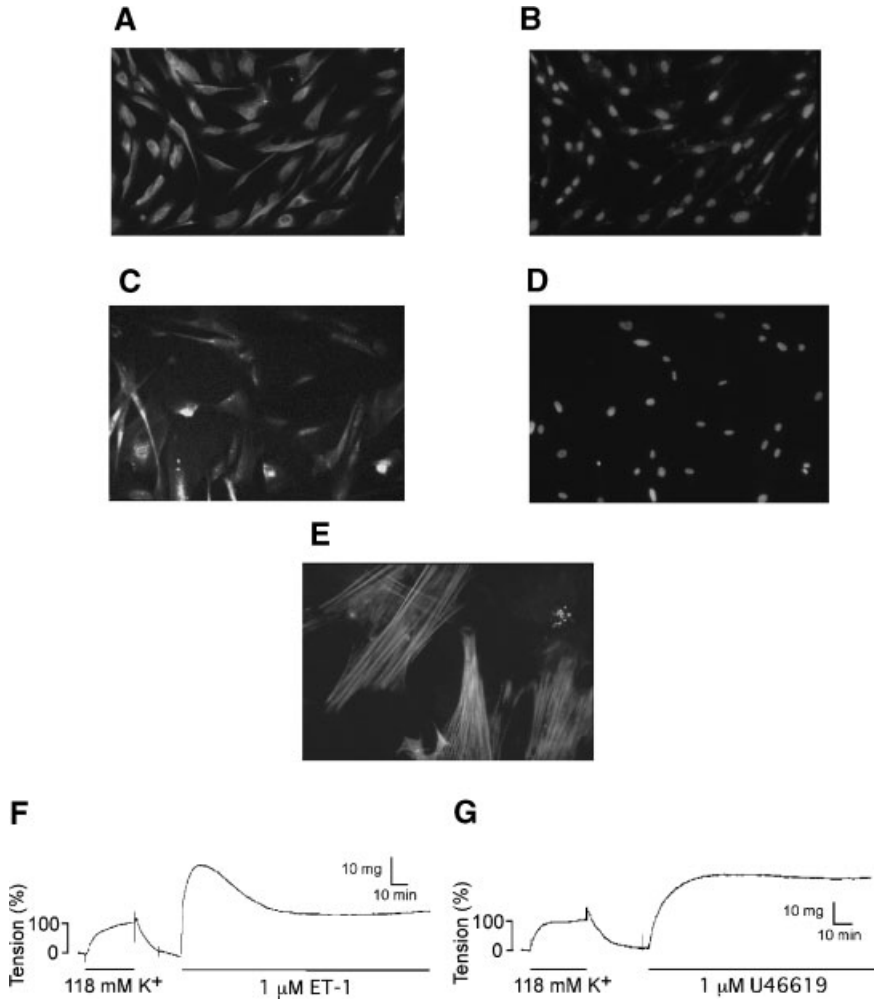


**Fig. 1.** Characterization and contractile activity of cultured human prostatic stromal cells (CHPSCs). **Panels A** and **B** show the representative immunofluorescence photographs (×400) of CHPSCs, which were double-stained with vimentin (A) and Hoechst 33258 (B). Almost all of the cells were positively stained with vimentin. **Panels C** and **D** show the representative immunofluorescence photographs (×400) of CHPSCs, which were double-stained with α-smooth muscle actin (C) and Hoechst 33258 (D). The percentages of smooth muscle cells were determined in 12 different fields of vision. About 90% of the cells were positively stained with α-smooth muscle actin antibody. **Panel E** is a magnified photograph (×630) of α -smooth muscle actin stained cells. The reconstituted rings made of CHPSCs produced the isometric tension developments in response to ET-1 (**F**) and U46619 (**G**). The resting and 118 mM $K^+$-induced contractions were assigned to be 0% and 100%, respectively. The traces shown are representative of 3–4 independent experiments.

$A_2$ analogue (Fig. 1F and G). The sustained phase of contraction induced by ET-1 or U46619 was $88 \pm 17\%$ ($n = 4$) or $198 \pm 27\%$ ($n = 3$), assigning the 118 mM $K^+$-induced contraction to be 100%.

### Effect of PDBu on Tension in Reconstituted Ring Made of CHPSCs

The application of 300 nM PDBu, a PKC activator, did not induce a contraction and had a rather relaxing effect (Fig. 2A). The level of tension after the application of PDBu was—$17 \pm 6\%$ ($n = 3$), assigning the resting and 118 mM $K^+$-induced contraction to be 0% and 100%, respectively. The negative value means that PDBu induced a relaxation. The application of 300 nM PDBu during a sustained contraction induced by ET-1 or U46619 also induced a relaxation (Fig. 2B, C). When the level of tension just before the application of PDBu was assigned to be 100%, PDBu decreased the ET-1 or U46619-induced tension by $84 \pm 8\%$ ($n = 4$) or $82 \pm 8\%$ ($n = 4$), respectively.

### Expression Levels of CPI-17 Protein in CHPSCs, Human BPH Tissue, and Rabbit Aorta

As shown in Figure 3A, CPI-17 protein was present in both CHPSCs and fresh human BPH

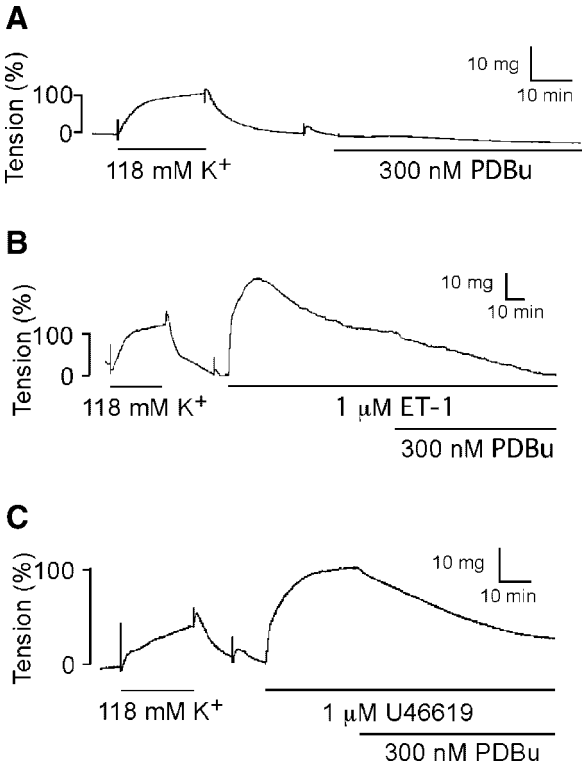


**Fig. 2.** Effect of PDBu on tension in the reconstituted ring made of CHPSCs. **Panels A**, **B**, and **C** show the functional effect of PDBu at a resting state (A) or during the sustained contraction induced by ET-1 (B) or U46619 (C). In panel A, the resting and 118 mM $K^+$-induced contractions were assigned to be 0% and 100%, respectively. In panels B and C, the sustained contraction induced by ET-1 (B) or U46619 (C) was assigned to be 100%. The traces shown are representative of four independent experiments.

tissue to the same extent. However, the expression levels of CPI-17 protein in these samples were significantly lower than that in the rabbit aorta (Fig. 3B).

### Effect of PDBu on Tension in Reconstituted Ring Made of CHPSCs Expressing Eyfp Alone or Over-Expressing CPI-17

Figure 4A shows a fluorescence photograph of CHPSCs expressing eYFP and CPI-17 fusion protein, using the baculovirus vector as described in the Materials and Methods. The ectopic expression of CPI-17 was also confirmed by immunoblotting (Fig. 4B). These results indicate that the baculovirus vector could also be used for the high efficiency gene transfer into CHPSCs. A similar result was obtained in case of other recombinant baculovirus expressing eYFP alone or eYFP + CPI-17 T38A (photographs not shown). For brevity, the cells expressing eYFP + X protein was shown as those expressing X.

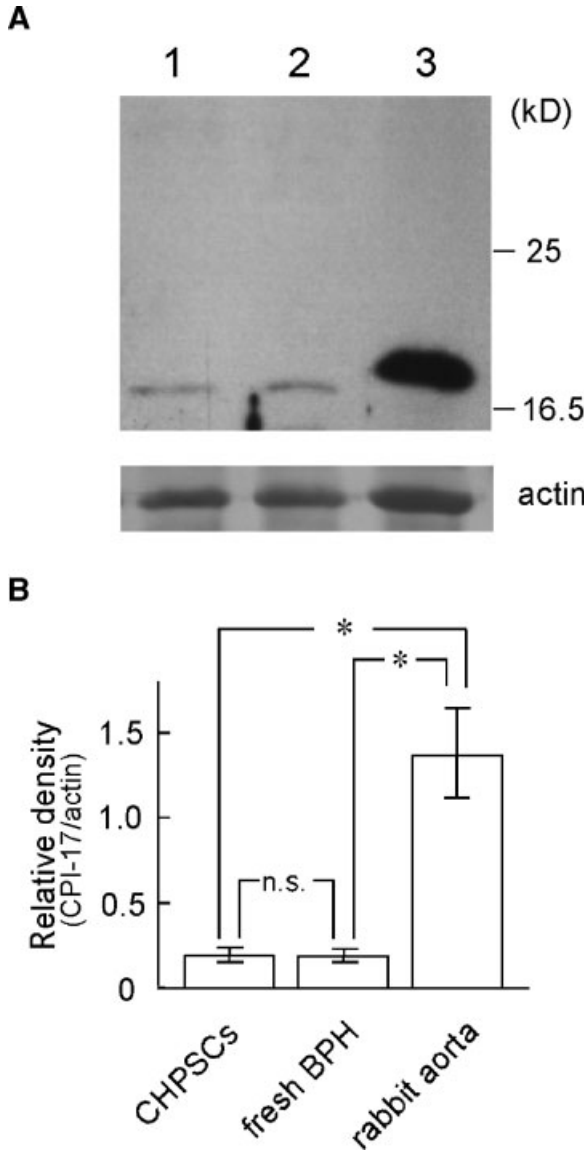


**Fig. 3.** Expression levels of CPI-17 protein in CHPSCs, fresh human BPH tissue and rabbit aorta. **Panel A** shows the expression of CPI-17 protein in CHPSCs (**lane 1**), fresh human BPH tissue (**lane 2**), and rabbit aorta (**lane 3**). A volume equal to 30 μg of total protein was applied in each lane. **Panel B** shows the densitometric analysis of the bands representing CPI-17 protein in CHPSCs, fresh human BPH tissue and rabbit aorta normalized to total actin band. Data are the mean ± SEM (n = 3). *; $P < 0.05$, ns; not significant.

The application of 300 nM PDBu to CHPSCs expressing eYFP alone did not induce a contraction and had a rather relaxing effect (Fig. 4C), a similar effect was observed in non-transfected CHPSCs (Fig. 2A). On the other hand, in CHPSCs over-expressing CPI-17, the application of 300 nM PDBu induced a large contraction (Fig. 4D), which was attenuated by the application of 3 μM GF109203X (38), a PKC

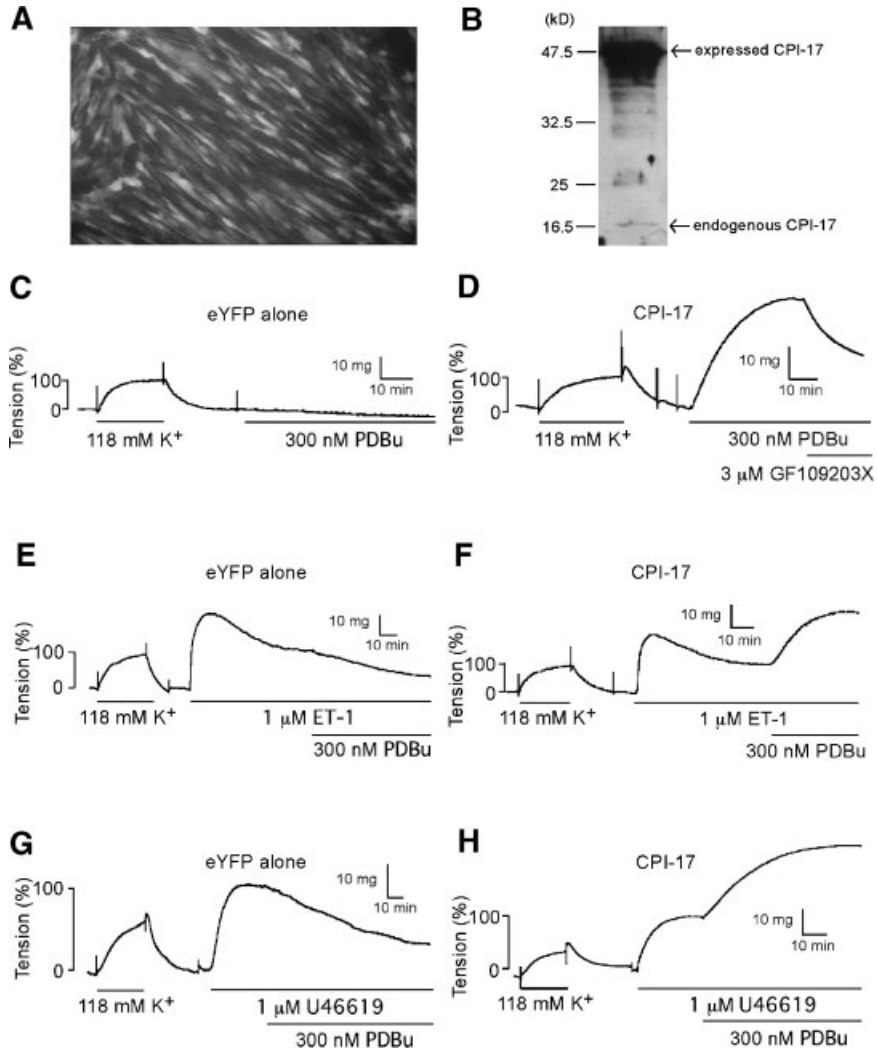


**Fig. 4.** Effect of PDBu on tension in the reconstituted ring made of CHPSCs expressing eYFP alone or over-expressing CPI-17. **Panel A** shows a fluorescence photograph of CHPSCs expressing eYFP and CPI-17 fusion protein. Magnification is ×100. In **panel B**, the expression of exogenous CPI-17 in CHPSCs was also confirmed by a Western blot analysis. **Panels C**, **E**, and **G** show the functional effect of PDBu at a resting state (C) or during a sustained contraction induced by ET-1 (E) or U46619 (G) in CHPSCs expressing eYFP alone. **Panels D**, **F**, and **H** show the functional effect of PDBu at a resting state (D) or during a sustained contraction induced by ET-1 (F) or U46619 (H) in CPI-17-over-expressing CHPSCs. In panels C and D, the resting and 118 mM $K^+$-induced contraction were assigned to be 0% and 100%, respectively. In panels E, F, G, and H, the sustained contraction induced by ET-1 (E and F) or U46619 (G and H) was assigned to be 100%. The traces shown are representative of 3-5 independent experiments.

inhibitor. The level of tension after the application of PDBu was $-18 \pm 9\%$ ($n = 3$) in CHPSCs expressing eYFP alone and $634 \pm 182\%$ ($n = 5$) in CHPSCs over-expressing CPI-17, assigning the resting and 118 mM $K^+$-induced contraction to be 0% and 100%.

In CHPSCs expressing eYFP alone, the application of PDBu during ET-1 or U46619-induced sustained contraction induced a relaxation (Fig. 4E and G), which was similar to the response observed in non-transfected rings (Fig. 2B and C). On the other hand, in CHPSCs over-expressing CPI-17, the application of PDBu during a sustained contraction induced by ET-1 or U46619 induced an additional contraction (Fig. 4F and H). When the level of tension just before the application of PDBu was assigned to be 100%, PDBu decreased the ET-1 or U46619-induced tension by $60 \pm 3\%$ ($n = 3$) or $62 \pm 6\%$ ($n = 4$) in CHPSCs expressing eYFP alone and increased the ET-1 or U46619-induced tension by $208 + 38\%$ ($n = 4$) or $232 + 87\%$ ($n = 4$) in CHPSCs over-expressing CPI-17, respectively.

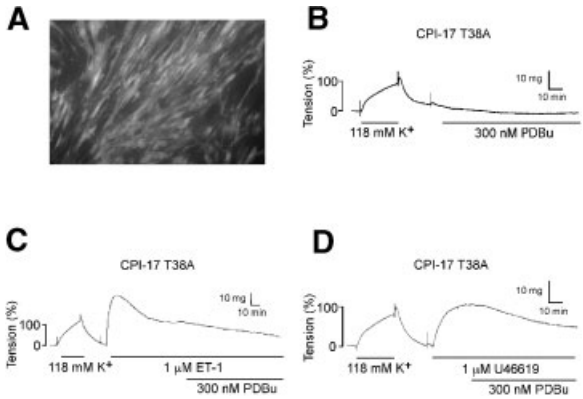


**Fig. 5.** Effect of PDBu on tension in the reconstituted ring made of CHPSCs expressing CPI-17 T38A. **Panel A** shows a fluorescence photograph of CHPSCs expressing eYFP and CPI-17 T38A fusion protein. Magnification is ×100. **Panels B**, **C**, and **D** show the functional effect of PDBu at a resting state (B) or during a sustained contraction induced by ET-1 (C) or U46619 (D). In panel B, the resting and 118 mM $K^+$-induced contraction were assigned to be 0% and 100%, respectively. In panels C and D, the sustained contraction induced by ET-1 (C) or U46619 (D) was assigned to be 100%. The traces shown are representative of 3–4 independent experiments.

### Effect of PDBu on Tension in Reconstituted Ring Made of CHPSCs Expressing CPI-17 T38A

Figure 5A shows a fluorescence photograph of CHPSCs expressing eYFP and CPI-17 T38A fusion protein. The application of 300 nM PDBu to CHPSCs expressing CPI-17 T38A did not induce any contraction (Fig. 5B). The level of tension after the application of PDBu was—$4 \pm 8\%$ ($n = 3$), assigning the resting and 118 mM $K^+$-induced contraction to be 0% and 100%, respectively. The application of 300 nM PDBu during a sustained contraction induced by ET-1 or U46619 induced a relaxation (Fig. 5C,D). When the level of tension just before the application of PDBu was assigned to be 100%, PDBu decreased the ET-1 or U46619-induced tension by $54 \pm 16\%$ ($n = 3$) or $81 \pm 18\%$ ($n = 4$), respectively. These functional effects of PDBu are closely similar to those observed in the non-transfectioned CHPSCs (Fig. 2).

### Agonists-Induced Contractions in Reconstituted Ring Made of CHPSCs Expressing eYFP Alone or Over-Expressing CPI-17

The sustained level of contraction of CHPSCs expressing eYFP alone or over-expressing CPI-17 was $90 \pm 9\%$ ($n = 9$) or $116 \pm 22\%$ ($n = 10$) during activation by 1 μM ET-1 (Fig. 6A and B) and $199 \pm 21\%$ ($n = 11$) or $150 \pm 28\%$ ($n = 10$) during activation by 1 μM U46619 (Fig. 6C and D), respectively, assigning the 118 mM $K^+$-induced contraction to be 100%. There was no significant difference in the level of contraction induced by ET-1 or U46619 between the CHPSCs expressing eYFP alone and over-expressing CPI-17, thus indicating that the over-expression of CPI-17 does not enhance the agonist-induced contraction. However, GF109203X [Toullec et al., 1991], a PKC inhibitor, significantly inhibited ET-1 ($38 \pm 6\%$; $n = 6$) or U46619 ($28 \pm 3\%$; $n = 6$)-induced contraction in CPI-17-over-expressing CHPSCs (Fig. 6B and D), while it had almost no effect in eYFP alone expressing CHPSCs (ET-1: $9 \pm 3\%$; $n = 6$, U46619: $5 \pm 2\%$; $n = 7$; Fig. 6A and C). On the other hand, Y27632 [Uehata et al., 1997], a Rho kinase inhibitor, almost completely inhibited ET-1 or U46619-induced contraction in both CHPSCs expressing eYFP alone and CPI-17-over-expressing CHPSCs (Figs. 6A–D). A Western blot analysis revealed that the expression level of RhoA was not changed in CPI-17-over-expressing CHPSCs compared with non-transfected CHPSCs or CHPSCs expressing eYFP alone (Fig. 7).

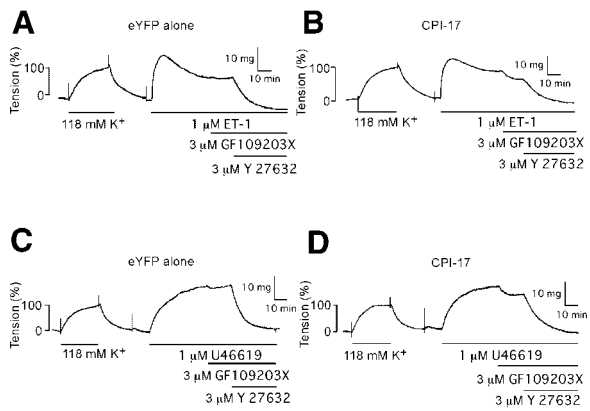


**Fig. 6.** Agonists-induced contractions of the reconstituted ring made of CHPSCs expressing eYFP alone or over-expressing CPI-17. **Panels A** and **B** show the ET-1-induced contractions and the effect of GF109203X and Y27632 on them in CHPSCs expressing eYFP alone (A) or over-expressing CPI-17 (B). **Panels C** and **D** show the U46619-induced contractions and the effect of GF109203X and Y27632 on them in CHPSCs expressing eYFP alone (C) or over-expressing CPI-17 (D). These agonists-induced contractions were not augmented even when CPI-17 was over-expressed. However, PKC inhibitor, GF109203X, significantly inhibited the agonists-induced contractions of the CPI-17-over-expressing CHPSCs, while it had almost no effect in CHPSCs expressing eYFP alone. The resting and 118 mM $K^+$-induced contraction were assigned to be 0% and 100%, respectively. The traces shown are representative of 6-7 independent experiments.

### Effect of PDBu on $Ca^{2+}$ Sensitivity in α-Toxin Permeabilized Preparation

In eYFP alone (Fig. 8A) or CPI-17 T38A (Fig. 8C) expressing CHPSCs, the application of 300 nM PDBu during 300 nM $Ca^{2+}$-induced sustained contraction induced a relaxation at a constant $[Ca^{2+}]_i$. However in CPI-17-over-expressing CHPSCs, PDBu induced a further contraction at a constant $[Ca^{2+}]_i$ (Fig. 8B). When the 300 nM $Ca^{2+}$-induced sustained contraction was assigned to be 100%, PDBu decreased the tension by $96 \pm 12\%$ ($n = 5$) in CHPSCs expressing eYFP alone or by $144 \pm 26\%$ ($n = 5$) in CPI-17 T38A expressing CHPSCs, while PDBu increased the tension by $33 \pm 8\%$ ($n = 6$) in CPI-17-over-expressing CHPSCs.

## DISCUSSION

In the present study, we prepared CHPSCs derived from BPH patients and investigated the functional role of PKC in CHPSCs contraction. The major findings were as follows: (1) PDBu, a PKC activator, induced a relaxation in CHPSCs ring preparation, while in CPI-17-over-expressing CHPSCs, PDBu induced a large contraction. (2) CPI-17 was expressed in both CHPSCs and fresh human BPH tissue at a similar level, which was much lower than that in the rabbit aorta. (3) The agonist-induced contraction did not become larger than expected even when CPI-17 was over-expressed. These results indicated that the activation of PKC in CHPSCs induces a relaxation probably due to the low expression level of CPI-17 and that PKC-CPI-17 pathway does not appear to play a major role in the agonist-induced contraction even when CPI-17 was over-expressed.

The human prostate is usually composed of epithelial cells, smooth muscle cells and fibroblasts. However, previous studies have demonstrated that the cultured cells derived from human prostate tissue was mainly composed of smooth muscle cells and fibroblasts [Kassen et al., 1996; Jansen et al., 2000]. Although epithelial cells also grow out from human prostatic tissue, they tend to disappear within a few days probably due to their low proliferation rate [Yazawa et al., 1994]. In this study, almost all cultured cells exhibited positive immunofluorescence staining for vimentin, an intermediate filament polypeptide that is specific for mesenchymal cells. In addition, about 90% of the cells were positively stained with anti-α-smooth muscle actin. These results indicated that the cultured cells used in this study would be predominantly composed of smooth muscle cells

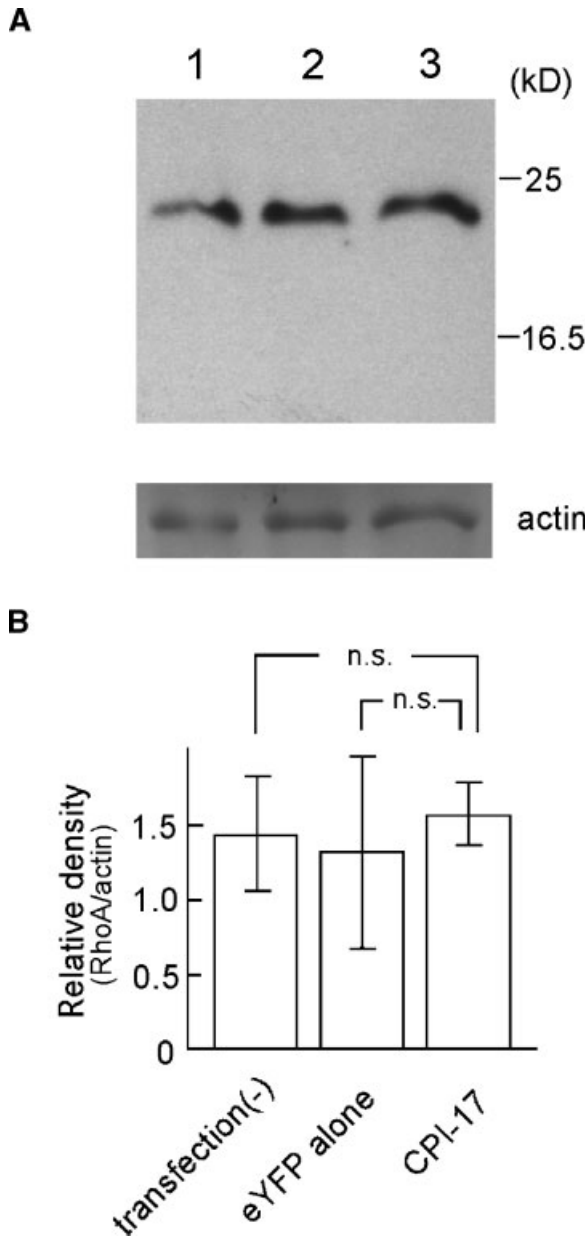


**Fig. 7.** Expression levels of RhoA protein in non-transfected, eYFP alone expressing and CPI-17 over-expressing CHPSCs. **Panel A** shows the expression of RhoA protein in non-transfected (**lane 1**), eYFP alone expressing (**lane 2**), and CPI-17-over-expressing (**lane 3**) CHPSCs. A volume equal to 10 μg of total protein was applied in each lane. **Panel B** shows the densitometric analysis of RhoA protein bands in non-transfected, eYFP alone expressing and CPI-17-over-expressing CHPSCs normalized to total actin bands. Data are the mean ± SEM ($n = 5$). n.s., not significant.

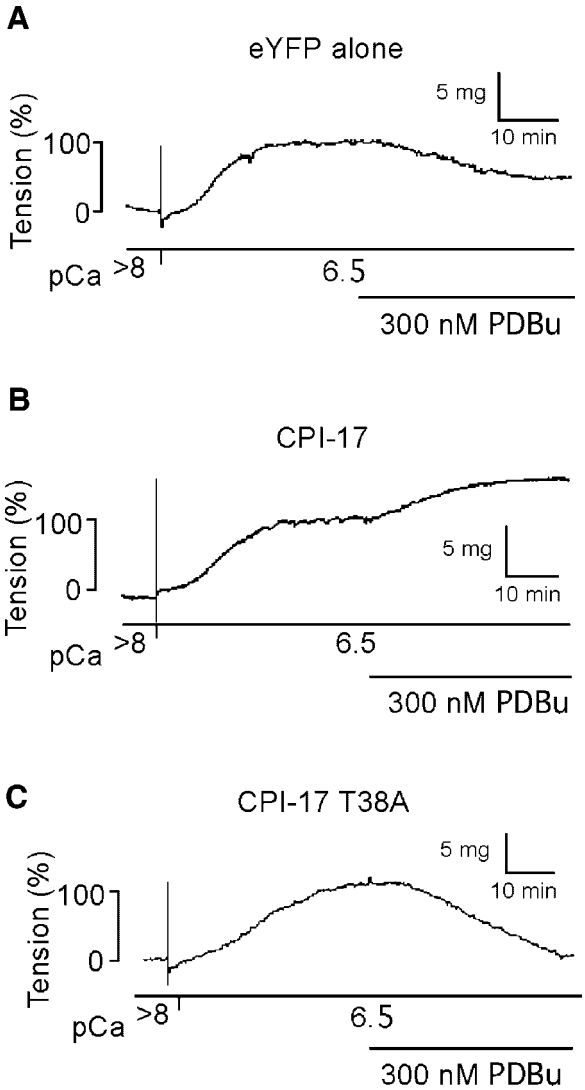


**Fig. 8.** Effect of PDBu on $Ca^{2+}$ sensitivity in eYFP alone, CPI-17 or CPI-17 T38A expressing CHPSCs. In eYFP alone (**A**) or CPI-17 T38A (**C**) expressing CHPSCs, PDBu induced a relaxation at a constant $[Ca^{2+}]_i$, while in CPI-17-over-expressing CHPSCs, PDBu induced a contraction at a constant $[Ca^{2+}]_i$ (**B**). The resting and 300 nM $Ca^{2+}$-induced sustained contractions were assigned to be 0% and 100%, respectively. The traces shown are representative of 5–6 independent experiments.

while the rest are probably fibroblasts. Recently, several studies reported the contractility of CHPSCs [Cook et al., 2002; Haynes et al., 2002] obtained from surgical human prostate specimens. However, in these reports, the contractility has been evaluated by the shortening of cell length. In the present study, we applied a collagen gel method to CHPSCs and thus were able to measure the isometric tension development of CHPSCs. To our knowledge, this is the first report that described the isometric tension development of CHPSCs.

As mentioned in the introduction, the effect of PKC activation on the contraction of human prostate remains controversial. We thus investigated the effect of PDBu, an activator of PKC, on the contraction of CHPSCs. As shown in Figure 2, PDBu induced a relaxation in CHPSCs ring preparations. The mechanisms for the PKC-induced inhibition of actin-activated myosin ATPase in smooth muscle have been previously investigated in several in-vitro studies. PKC has been reported to phosphorylate MLC at Ser-1, Ser-2, and Thr-9 distinct from MLC kinase (MLCK) sites [Nishikawa et al., 1983; Bengur et al., 1987; Ikebe et al., 1987] and the phosphorylation of these PKC sites causes a decrease in the actin-activated myosin ATPase activity. Furthermore, MLCK has been demonstrated to be phosphorylated by PKC, which resulted in the reduced affinity of MLCK for calmodulin [Ikebe et al., 1985; Nishikawa et al., 1985]. In support of these in-vitro studies, the application of PKC enzyme into the detergent-skinned fiber, in which CPI-17 protein leaked out (as discussed below), was shown to induce a relaxation of the contractile force levels induced by submaximal $Ca^{2+}$ [Inagaki et al., 1987; Parente et al., 1992]. In addition, the stimulation of PKC has been reported to induce a relaxation in several intact tissues [Baraban et al., 1985; Menkes et al., 1986; Kim et al., 1996]. It was thus speculated that these mechanisms might contribute to the PDBu-induced relaxation of CHPSCs.

In contrast to the PDBu-induced relaxation of CHPSCs, it is well known that PDBu induces a contraction in intact and the receptor-coupled permeabilized smooth muscle preparations [Rasmussen et al., 1984; Nishimura et al., 1989; Masuo et al., 1994]. We thus wondered why PDBu induces a relaxation in CHPSCs unlike other smooth muscle preparations. Although the PKC-mediated inhibitory effect on the contractile signal transduction pathways has been well characterized as mentioned above, the mechanism for the smooth muscle contraction during PKC activation has long been unidentified. However, it has been recently reported that CPI-17, which was thought to be specifically expressed in smooth muscle, inhibits MLCP when phosphorylated by PKC

at Thr-38 [Eto et al., 1997]. Kitazawa et al. [1999] succeeded in reconstituting the contractile $Ca^{2+}$ sensitization by PKC in a demembranated (with Triton X-100) preparation by replenishing PKC and CPI-17. The reason why the application of PKC enzyme into such detergent-skinned fiber failed to induce contraction in previous studies [Inagaki et al., 1987; Parente et al., 1992] was explained by the leakage of CPI-17 protein from the detergent-skinned fibers [Kitazawa et al., 1999]. Furthermore, it has also been reported that the magnitude of phorbol ester induced contraction is positively correlated with the extent of expression of CPI-17 [Woodsome et al., 2001]. According to these considerations, we speculated that PKC may have two distinct effects on the contractility of smooth muscle and total functional effect of PKC-activation may therefore depend on the balance between the contractile and relaxing pathways, which might be determined by the expression level of CPI-17 in each tissue. We thus performed a Western blot analysis and found that the expression level of CPI-17 in CHPSCs was very low compared to that in the rabbit aorta, a representative tissue of tonic arterial smooth muscle, which has been reported to contain an abundant amount of CPI-17 protein and can develop a large contraction with the application of PDBu [Woodsome et al., 2001]. We thus speculated that the PDBu-induced relaxation of CHPSCs might be due to the predominance of the relaxing pathway over the contractile one because of a low expression level of CPI-17 in CHPSCs. In a Western blot analysis, human CPI-17 proteins were detected at a lower molecular weight on SDS gels compared with the rabbit aorta protein. This observation was also reported in the previous study [Wang et al., 2002]. Although the precise mechanisms were not clarified, another isoform of CPI-17 (13.5 kDa) as reported in human aorta [Yamawaki et al., 2001] may exist in human tissue samples and may be involved in this observation.

In order to confirm the above speculation and also to rule out the possibility that the pathway for the activation of CPI-17 might be lacking in CHPSCs, we next over-expressed CPI-17 in the CHPSCs and examined the contractile activity to PDBu as well as to the selected contractile agonists. As shown in Figure 4, PDBu induced a large contraction in CPI-17-over-expressed CHPSCs, while PDBu induced a relaxation in CHPSCs expressing eYFP alone or CPI-17 T38A. These results supported the idea that PDBu-induced relaxation of CHPSCs is due to the low expression of CPI-17 in CHPSCs, but not due to the absence of the pathway for the activation of CPI-17. While this manuscript is being prepared, Kitazawa et al. [2004] reported that introduction of recombinant CPI-17 in β-estin-permeabilized chicken artery, which lacks CPI-17, converted PKC-mediated relaxation into contraction. Our data obtained by the gene transfer technique are consistent with their results.

Although there might be an underestimate of CPI-17 expression level of the smooth muscle cells in the fresh BPH tissue due to the dilution by a higher percentage of fibroblasts and epithelial cells in the fresh BPH tissue than in CHPSCs, the expression level of CPI-17 in fresh BPH tissue was almost the same level as that in CHPSCs. Therefore, it can be speculated that the activation of PKC might also induce a relaxation in fresh human BPH tissue. In agreement with this, Marshall et al. [1999] reported that PKC inhibitor did not alter the contraction to noradrenaline and human prostatic tissue specimens showed no contraction after the administration of PDBu.

Since agonist stimulation is supposed to involve PKC activation, we expected that the agonist-induced contraction would be much larger than the contraction induced by high external $K^+$ solution when CPI-17 was over-expressed. However, the contraction induced by ET-1 or U46619 in CHPSCs over-expressing CPI-17 was as large as that in CHPSCs expressing eYFP alone. In order to assess the relative contribution of PKC to the agonist-induced contraction, we applied a PKC inhibitor, GF109203X [Toullec et al., 1991], which could effectively inhibit the PDBu-induced contraction of the CPI-17-over-expressing CHPSCs. As shown in Figure 6B and D, GF109203X significantly but only slightly inhibited the U46619-induced contraction of the CPI-17-over-expressing CHPSCs, while it had almost no effect in CHPSCs expressing eYFP alone. We thus considered that the agonist-induced activation of PKC is coupled with the activation of CPI-17 in CHPSCs over-expressing CPI-17. However, it was also concluded that the PKC-CPI-17 pathway does not appear to play a major role in the agonist-induced contraction even when CPI-17 was over-expressed.

The receptor stimulation by a contractile agonist is coupled not only with the PKC-CPI-17 pathway but also with RhoA-Rho kinase pathway [Kimura et al., 1996]. The latter pathway appeared to play a major role in the agonist-induced contraction of CHPSCs, because Y27632 [Uehata et al., 1997], a Rho kinase inhibitor, was much more effective than GF109203X in inhibiting the agonist-induced contraction of both CPI-17-over-expressed and eYFP alone expressing CHPSCs. We thus examined the expression of RhoA in CHPSCs over-expressing CPI-17. As shown in Figure 7, the over-expression of CPI-17 did not affect the expression level of RhoA. These results were consistent with the idea that the RhoA-Rho kinase pathway, but not the PKC-CPI-17 pathway, may play a major role in the agonist-induced contraction of CHPSCs even when CPI-17 was over-expressed.

The contraction of smooth muscle is known to be regulated not only by intracellular $Ca^{2+}$, but also by the $Ca^{2+}$ sensitivity of the contractile apparatus [Nishimura et al., 1988]. To examine the effect of PDBu on $Ca^{2+}$ sensitivity in CHPSCs, we applied α-toxin-mediated permeabilization to this ring preparation. As shown in Figure 8, the application of PDBu decreased the $Ca^{2+}$ sensitivity of CHPSCs expressing eYFP alone or CPI-17 T38A, while in CPI-17-over-expressing CHPSCs, PDBu increased $Ca^{2+}$ sensitivity. These results indicated that the PDBu-induced increase or decrease in $Ca^{2+}$ sensitivity would also be determined by the expression level of CPI-17.

In the present study, we applied the collagen gel method to CHPSCs in order to measure the isometric tension development. Considering the difficulty of obtaining a sufficient amount of fresh human prostate tissue, the availability of CHPSCs for an isometric tension study would provide a valuable resource for studying the prostatic physiology. However, further refinements will be required to obtain model systems that fully mimic the in vivo processes, because our CHPSCs did not respond to α-stimulant, a major contractile agent in human prostate [Caine et al., 1975; Hedlund et al., 1985], probably due to some change in the receptor phenotype during culture. In addition, baculovirus vector was very useful for transferring the target genes into CHPSCs. Therefore, the combined use of these methods would also be a valuable tool for exploring the signal transduction pathway in contraction of human prostate tissue.

In conclusion, the results obtained in the present study indicated that PDBu induces a relaxation in CHPSCs probably due to low expression level of CPI-17. The expression level of CPI-17 in each tissue may therefore be a key factor for the functional effect (contraction or relaxation) of PKC-activation. Although the over-expression of CPI-17 converted PDBu-induced relaxation into contraction due to an increase in $Ca^{2+}$ sensitivity, the PKC-CPI-17 pathway does not appear to play a major role in the agonist-induced contraction even when CPI-17 was overexpressed.

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